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BALTIMORE	
School of Hygiene and Public Health	December 19, 1951
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University of Minnesota	December 19, 1951
SOUTHERN CALIFORNIA	
Cedars of Lebanon Hospital, Los Angeles	December 13, 1951

Resistance and Cross Resistance of Bacteria to Nitrofurans. (19321)

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It has been shown that many nitrofurans have a broad antibacterial spectrum(1-4). Green(5) has demonstrated that *Micrococcus pyogenes var. aureus* can develop limited resistance to 5-nitro-2-furaldehyde semicarbazone (Furacin*). The present study is concerned with development of bacterial resistance and cross resistance to nitrofurans of varied chemical structure.

The nitrofurans used in this study were characterized by a nitro group in the 5-position of the furan ring and a semicarbazone, semioxamazone, or closely related side chain in the 2-position.† Most of these nitrofurans have a basic structure correlated with chemotherapeutic activity(4).

Experimental. For the preliminary phase of the study of resistance development, three organisms: *Escherichia coli* — A.T.C. No. 6522, *M. pyogenes var. aureus*—F.D.A No. 209, and *Streptococcus faecalis*—A.T.C. No. 6057, were habituated to a selected nitrofuran by exposing the bacteria to 2-fold increasing concentrations in Difco nutrient broth using smaller increments as limiting resistance was approached. The starting inoculum was one loopful (4 mm loop of 23 gauge platinum wire) of the 24-hour culture of the test bacteria grown in broth. Subsequently, a loop inoculum was taken from the tube containing the highest concentration of the nitrofuran in which definite visible growth occurred. Density of growth for all 3 organisms was reduced considerably after the first few subcultures under the influence of the nitrofuran and 24-hour transfers were changed

* Brand of nitrofurazone N.N.R.

† The compounds were synthesized by the Division of Chemistry of Eaton Laboratories.

TABLE I. Development of Resistance to 5-Nitro-2-Furaldehyde 2-(2-Hydroxyethyl) Semicarbazone (NF-67) in Broth.

Test organism	Conc. NF-67 mg/l in which visible growth occurred*		Degree of resistance development	No. of subcultures
	at start	at end		
S. faecalis	31	400	13x	6
M. aureus	9	200	22x	8
E. coli	19	400	21x	9

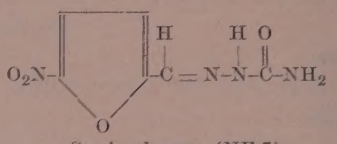
* Loop inocula.

to 48 hours to allow suitable growth. The resistance thus obtained by 3 organisms against a selected nitrofuran (NF-67) is shown in Table I.

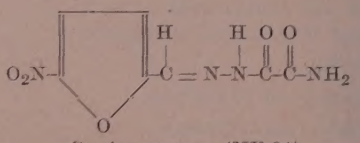
After the limiting resistance had been reached by the loop method, substitution of a larger inoculum (0.1 ml) in place of the loop inoculum (about 0.01 ml) resulted in attenuated growth in the case of *E. coli* and *M. aureus* up to twice the above concentrations. Therefore 0.1 ml inocula were used in all subsequent studies in developing resistant organisms.

Although resistance to NF-67 occurred in the 3 bacteria tested, its development was definitely slower (about 20x in 8 subcultures) than observed by Klimek *et al.*(6) in the development of resistance of *M. aureus* to penicillin (about 80x in 8 subcultures) and to streptomycin (about 200x in 8 subcultures).

E. coli. The development of resistance by *E. coli* to various nitrofurans was investigated next. The nitrofurans studied and their structure appear in Table II. The water-solubility of the nitrofurans studied is given since it is a limiting factor in some cases. The data in columns 4 and 5 show that *E. coli* can develop resistance to all nitrofurans studied. Of importance is the fact that, where solubility permits, a limiting concentration is reached to which bacteria cannot readily develop resistance. It was found that bacteria resistant to single semicarbazones such as NF-7 (Furacin), NF-61, and NF-67 were cross resistant to other semicarbazones. However these organisms were still susceptible to the semioxamazones, NF-84 and NF-89. The structure of the simplest member of each class is as follows:



Semicarbazone (NF-7)

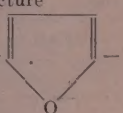


Semioxamazone (NF-84)

A strain of *E. coli* highly resistant to a typical semicarbazone (NF-67) and a strain highly resistant to a typical semioxamazone (NF-84) were now titrated for possible cross resistance to a series of nitrofurans chosen for varying structure, suitable solubility and degree of antibacterial activity. The last two columns of Table II indicate, on the basis of cross resistance, that the nitrofurans studied fall into two groups. *E. coli* resistant to the semicarbazone NF-67 is cross resistant to a series of nitrofurans which we have arbitrarily classified as Type I compounds. This same NF-67-resistant *E. coli* is, however, susceptible to another series of nitrofurans which we term Type II compounds.

From inspection of the structural formulas of the nitrofurans in Table II it will be noted that the Type II nitrofurans all differ from the Type I nitrofurans by having a carbon atom between the first carbonyl carbon and the terminal group of the side chain. On the basis of this observation the study of cross resistance was extended by developing strains of *E. coli* which were highly resistant to each of 4 individual nitrofurans of the Type I series and to 3 of the Type II series, as shown in the

TABLE II. Resistance and Cross Resistance of *E. coli* to Nitrofurans of Varied Structure.

Nitrofuran No. (NF)	Structure ($R = O_2N$–  –)	Solubility in water, mg/l¶	Resistance*		Cross resistance*	
			Parent strain	Resistant strain	Strain resistant to NF-67†	Strain resistant to NF-84‡
	Type I					
7	R—CH = N·NH·CO·NH_2	210	12.5	75	100 C	100 C§
61	R—CH = N·N(·CH_3)·CO·NH_2	317	6.5	100	100 C	100 C
62	R—CH = N·NH·CO·NH·CH_3	670	25		200 C	200 C
64	R—CH = N·NH·CO·CH_3	135	11.2		45 C	
65	R—CH = N·NH·CO·NH·CO·NH_2	60	8.75		70 C	
67	R—CH = N·N(·CH_2CH_2OH)·CO·NH_2	1250	12.5	600	—	200 C
106	R—C(·CH_2OH) = N·NH·CO·NH_2	275	12.2		196 C	
131	R—C(·CH_2·CH_2–N⟨ ⟩·HCl)=N·NH·CO·NH_2	12100	50		400 C	200 C
145	R—CH = N·NH·CO·NH·CH_2·CH_2·OH	1030	38.3		307 C	
147	R—C(·CH_3) = N·NH·CO·NH·CH_2·CH_2·OH	157	58.6		234 C§	234 C§
148	R—CH = N·N(·CH_3)·CO·NH·CH_3	600	24.6		395 C	
153	R—CH = N·N(·CH_2·CO)·CO·NH	125‖	4.2	200	162 C	137 C
156	R—CH = N·N(·CH_2·CHOH·CH_3)·CO·NH_2	420	19.3		620 C§	
	Type II					
69	R—CH = N·NH·CO·CH_2·NH_2·HCl	60400	12.1	80	24 N	100 C§
82	R—CH = N·NH·CO·CO·OC_2H_5	220	62.5		125 N	
84	R—CH = N·NH·CO·CO·NH_2	120	9.37	100	12.5 N	
89	R—CH = N·NH·CO·CO·NH·CH_2CH_2OH	140	11.2		22.4 N	
114	R—CH = N·NH·CO·CH_2·$N(CH_3)_3$·Cl	89000	23.9	200	23.9 N	200 C

* Highest concentration in mg/l of nitrofuran permitting visible growth in 24-48 hr. Median value used where determination was run repeatedly.

† Organisms in which resistance to 600 mg NF-67 per 1 had been developed. C = cross resistance, N = non-cross resistance.

‡ Organisms in which resistance to 100 mg NF-84 per 1 had been developed.

§ Highest conc. tried.

‖ At pH 5 for this solubility. There is a marked increase in solubility with an increase of pH with this compound: Furadantin, brand of nitrofurantoin.

¶ Usually higher in culture media.

vertical column of Table III, grouped according to type. This tabular form of presentation is similar to that used by McIntosh and Selbie(7) for convenience in evaluating the entire resistance picture. These seven resistant strains were then individually titrated

TABLE III. Degree of Resistance and of Cross Resistance of *E. coli* to Certain Nitrofurans.

E. coli resistant to nitrofurans below	Degree of resistance*						
	Type I				Type II		
	NF-7	NF-61	NF-67	NF-153	NF-69	NF-84	NF-114
	Type I						
NF- 7	*6*	16	8	32	2	1	1
61	4	*10*	32	16	2	1	1
67	8	16	*32*	32	2	1	1
153	4	4	8	*37*	2	1	1
	Type II						
69	2	8	16	32	*8*	2	1
84	16	16	32	32	16	*8*	8
114	2	2	4	4	8	2	*8*

* Values = highest nitrofuran conc. in which resistant strain grew divided by highest concentration parent strain grew tested concurrently.

against each of the nitrofurans in the top horizontal column. For the experiments presented in this table, titrations were done by serial dilution except in a few cases where intermediate dilutions were used. All values in the table were obtained by dividing the highest nitrofuran concentration in which the resistant strain could grow, by the highest concentration in which the parent susceptible strain grew when tested concurrently. The seven italicized figures on the diagonal represent the degree of resistance displayed by the resistant *E. coli* to the specific nitrofuran to which it had become habituated, as compared to the susceptible parent organism at the time of the study.

Data in the upper left quadrant show that a strain of *E. coli* resistant to any Type I nitrofuran is cross resistant to all other Type I nitrofurans (all numbers being 4 or greater). This phenomenon, which we term *reciprocal cross resistance*, occurs with all compounds of Type I studied to date. Examination of the upper right quadrant of Table III shows in a clear-cut manner that *E. coli* strains resistant to Type I nitrofurans are not resistant to Type II nitrofurans, since regardless of the strength of resistance that *E. coli* had developed to a Type I nitrofuran, it was still about as susceptible to a Type II nitrofuran as the parent non-resistant organism. However, from the lower left quadrant is can be readily observed that *E. coli* strains resistant to Type II compounds are, in most cases, also resistant to Type I compounds. Three exceptions may be noted: NF-69-resistant strain is susceptible to NF-7, and the NF-114-resistant strain to NF-7, and NF-114-resistant strain to NF-61. These 3 pairs are also reciprocally non-cross resistant as may be noted from data in the upper right quadrant—*i.e.*, *E. coli* resistant to either member of these pairs is susceptible to the other member. Careful examination of the lower right quadrant indicates that, although there are cases of cross resistance and non-cross resistance among compounds of Type II, in no case does reciprocal cross resistance or reciprocal non-cross resistance occur within this type. Upon examination of the table as a whole, it is to be noted that *E. coli* resistant to any of the other nitrofurans is susceptible to NF-84; moreover, *E. coli* resistant to NF-84 is cross resistant to all other nitrofurans studied.

Streptococcus pyogenes. Supplementary studies of the resistance behavior of a gram-positive organism, *Strep. pyogenes*, were now made. This organism was chosen since it has been used in our laboratories for *in vivo* chemotherapy studies with the nitrofurans. A different culture medium, brain heart infusion, was required. In all other respects the technic for this work was the same as described for the investigation of *E. coli*. These studies were carried out using representative nitrofurans NF-7 and NF-67 from Type I, and NF-84 and NF-114 from Type II. The data from this study are presented in Table IV. Examination of this table in the same manner as Table III indicates that the following generalizations can be made for the organisms studied. 1. Bacteria resistant to any one member of Type I nitrofurans are cross resistant to all nitrofurans of Type I.

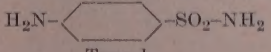
TABLE IV. Degree of Resistance and of Cross Resistance of *Strep. pyogenes* to Certain Nitrofurans.

Strep. pyogenes resistant to nitrofurans below	Degree of resistance*			
	Type I		Type II	
	NF-7	NF-67	NF-84	NF-114
	Type I			
NF- 7	*8*	20	1	1
67	8	*20*	1	1
	Type II			
84	8	8	*3*	8
114	8	5	2	*4*

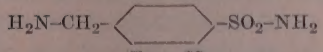
* See footnote Table III.

2. Bacteria resistant to any one member of Type I nitrofurans are susceptible to any member of Type II nitrofurans. 3. Only nitrofurans of Type I show reciprocal cross resistance. It is also indicated that cases of reciprocal non-cross resistance may occur between Type I and Type II nitrofurans. No suitable nitrofuran was available to determine whether the presence of two carbon atoms between the carbonyl carbon and the terminal group would produce a class of nitrofurans differing in resistance behavior from those of Type I and Type II.

Clostridium perfringens. A structural analogy has been noted between our two nitrofuran types and the sulfonamides (Type I) and a closely related class of compounds (Type II) studied by both Klarer(8) and Domagk(9).



Type I



Type II

In their Type II compounds, the amino group (or substituted amino group) is separated from the benzene ring by a CH_2 group. Their Type II compounds differ markedly from the sulfonamides in being highly active against the anaerobic organisms of the Clostridium group and in not being inhibited by p-aminobenzoic acid. While it was known that Furacin (NF-7) was moderately active against the Clostridium group of anaerobic bacteria, no such data were available on our Type II nitrofurans. It was therefore planned to titrate 2 typical Type I nitrofurans concurrently with 2 typical Type II nitrofurans against *Cl. perfringens*. The common method of using thioglycollate in the medium for the maintenance of anaerobic conditions was not permissible since the concentration required led to nitrofuran destruction, probably by the reducing action of thioglycollate. The use of freshly prepared brain-heart infusion broth with 0.1% agar coupled with vacuum or nitrogen gas for maintenance of anaerobic conditions permitted an evaluation of these two types of nitrofurans. Type I nitrofurans NF-7 and NF-67 prevented growth from a heavy inoculum (0.1 cc of 20-hour culture) of *Cl. perfringens* at 50 and 25 mg per liter respectively. Comparatively, Type II nitrofurans NF-84 and NF-69 prevented growth only at levels greater than 50 mg and 150 mg per liter respectively. Contrary to our expectation, it is clearly shown that the Type II nitrofurans have no greater antibacterial activity than has the Type I group against this anaerobe.

Nitrofuran-antibiotic cross resistance. A study by Green and Mudd(10) indicates that bacteria with developed resistance to sulfonamides, streptomycin, and penicillin, remain susceptible to Furacin (NF-7). In their work *M. aureus* strains resistant to either sulfathiazole or penicillin were as susceptible to Furacin as the parent strains. *E. coli* and several other organisms resistant to streptomycin were not resistant to Furacin. The present study covers similar observations with chloramphenicol and aureomycin. A chloramphenicol-resistant strain of the *E. coli* used throughout these studies was developed in the usual manner (from 2 mg up to 100 mg per liter) and tested for resistance to the individual nitrofurans NF-7, NF-67, NF-84, and NF-114. *E. coli* resistant to chloramphenicol remained as susceptible to our series of four representative nitrofurans as the parent strain. An aureomycin-resistant strain of the same organism was then developed (from 0.6 mg per liter up to 10 mg per liter) and tested for resistance to this same series of nitrofurans. This aureomycin-resistant strain was also as susceptible to the nitrofurans as the parent strain. Strains of *E. coli* resistant to NF-7, NF-67, NF-84, and NF-114 were tested for

concomitant resistance to chloramphenicol and to aureomycin. No cross resistance was indicated, with the possible exception of a slight cross resistance (4x) of the 2-(2-hydroxy ethyl) substituted semicarbazone (NF-67)-resistant strain to chloramphenicol.

These studies, added to those of Green and Mudd(10), indicate that those bacteria studied, with developed resistance to chloramphenicol, aureomycin, penicillin, streptomycin, and sulfonamides, retain unaltered their susceptibility to the nitrofurans.

Summary. (1) Bacteria can develop only a limited resistance to the nitrofurans *in vitro*. On the basis of a bacterial cross resistance study of a series of eighteen nitrofurans, it was found that the compounds investigated could be divided into two classes. The second class differs in chemical structure by having a carbon atom between the carbonyl group and the terminal group of the nitrofuran side-chain. Bacteria becoming resistant to the first class are reciprocally cross resistant to all members of this class but remain susceptible to nitrofurans of the second class. (2) Bacteria in which resistance was developed to chloramphenicol or to aureomycin remain susceptible to the nitrofurans. The converse is also true.

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Particulate Component of the Plasma of Fowls with Avian Lymphomatosis.* (19322)

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The avian leukosis complex consists in two chief pathological types of neoplastic disease (1). One of these is an "intravascular" process characterized by the occurrence in the blood stream of large numbers of primitive cells of erythroblastic or myeloblastic characters. The other type, lymphomatosis, is manifested in various forms dependent on localized or widespread overgrowths of primitive cells of the lymphoid type which may reach the circulation in only small numbers or not at all. Evidence of the viral etiology of both types of the complex has been demonstrated repeatedly, and a principal problem in the study of the complex is the possible relationships of the respective causative agents.

One approach to this problem is the purification of the viruses associated with the diverse forms of the complex and the direct comparison of their physical, chemical, and immunological properties. Progress in this direction has been made in recent work(2,3) in the purification and study of the virus of erythromyeloblastic leukosis which has been found to be present in large amounts in the plasma of affected chicks. It has been shown (4), also, that the infectious agent of some

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forms of lymphomatosis is likewise present in the plasma of chickens with this form of the complex and can be sedimented by centrifugation(5). In the present work, studies have been made on such plasmas by ultracentrifugal and electron microscopic procedures, and the results have revealed particles of characters differing from and in numbers far greater than those of any other particles of comparable size in the plasma of normal chickens. The findings in this work are described briefly here.

Materials and methods. The plasmas investigated were obtained from chickens affected with the RPL 12 strain(6,7) initially classified(4) as visceral lymphomatosis and from other chickens judged to be normal. The condition has been readily transmissible(7) by means of cell-free filtrates of both tumor extracts and plasma. In order to obtain the plasma as nearly as possible at the height of the disease in the donor, repeated examinations were made of blood smears. The criterion employed to judge the time of bleeding, in addition to clinical evidence of the disease, was the appearance of a few primitive cells of the lymphoid type in the blood stream. Blood was drawn into heparin as previously described(2), and the cells were removed by centrifugation. All of the plasmas had been frozen from 9 to 19 days before the studies were made. Immediately before use, the frozen plasma was thawed and spun in the cold room at 2000 X g for 15 minutes. The investigation consisted in the search of 3 series of individual plasmas for particulate material which might represent the virus. For this, the technic of sedimenting the particles onto an agar surface was employed, as in similar studies on erythromyeloblastic leukosis(3). Counts of the particles were made from micrographs of particles spun down from plasma diluted 1-10 in all instances. To obtain purified concentrates, plasma was spun at 20,000 X g for 60 minutes, and the sediment was resuspended in Ringer's solution. The process was repeated a second time by spinning at 20,000 X g for 30 minutes, after which the final concentrate, taken up in Ringer's solution, was spun in the horizontal centrifuge at 2,000 X g for 10 minutes, leaving the particles suspended in the supernatant fluid. Electron micrographs were obtained (3) by drying this particle suspension on an agar surface, fixing the particles with osmic acid and coating the particles with chromium.

TABLE I.

Donor	Interval since inoculation, days	Pathology	Particles/ml plasma ($\times 10^7$)
M 130 Q_2	42*	L†	32
1415 D	105	L and O	29
"	107	L and O	47
119 R_2	66	L	4
1305 A	110	L and O	160
727 B_3	98	L	5
119 I_2	77	L	1

* All chicks were inoculated at 1 day of age.
† The lymphoid involvement in all cases was confined to vascular networks (see text). L = Lymphomatosis; O = Osteopetrosis.

Results. In all of one series of plasmas from affected birds, all in advanced stages of the disease as determined at necropsy, there were observed significant and, in some instances, as shown in Table I, very large numbers of particles of similar characters with respect to size and shape. The characters of the particles are illustrated in Fig. 1(A) and (B) which reveal the presence of nearly spherical or spheroidal entities of 70 to 160 mμ, with an average of about 117 mμ diameter. The similarity, both in size and appearance, of these particles to the virus of the erythromyeloblastic type of leukosis obtained and portrayed by the same technic (Fig. 6 of (3)) is clearly evident.

The character of the material obtained by the ultracentrifugal procedures with 5 ml of a third plasma, M 1305 A, are shown in Fig. 2. It is evident that a high degree of purification was not effected since many masses apparently not agglomerates of the particles were present. It is still clear, nevertheless, that this small volume of plasma contained very large amounts of the particulate component identical in appearance with the particles shown in Fig. 1 (A) and (B). Again the particles seen in this concentrate are essentially indistinguishable from those (Fig. 4 of (3)) in the concentrates of plasmas from birds with erythromyeloblastic leukosis.

Examination of a second series of 6 plasmas from birds which had been inoculated with

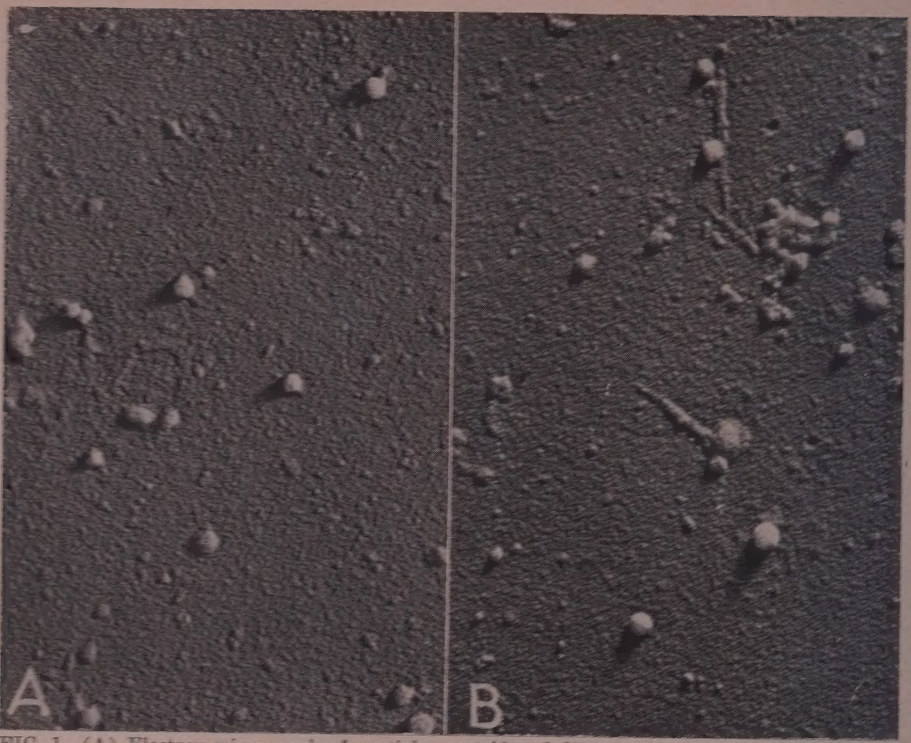

FIG. 1. (A) Electron micrograph of particles centrifuged directly from plasma M 1415 D and (B) the micrograph of particles obtained in the same way from plasma M 130 Q_2, from which the particles were counted as shown in Table I. Magnification 24000 ×.

lymphomatous material revealed parallel results in but a single case. In four instances, no particles of this sort were seen. An occasional particle was observed in one case. In only one plasma of this series was there a significant number of particles observed, but here the number was high in the range of Table I. The bird providing this plasma was also the only one of this series that had unquestionably gross pathology at necropsy; all others were diagnosed by microscopic examination and presumably were cases very early in the lymphomatous process. Thus, it would appear that the differences obtained between the first and second series of plasmas were related, in part, if not altogether, to the stage of the disease.

Plasmas from 6 birds regarded as normal were examined in the present work, and many others have been studied in the investigation of the virus of erythromyeloblastic leukosis. In only one instance of the present series were particles observed in significant numbers; in this case, however, the characters of the particles differed somewhat from those of the entities present in the plasmas of Table I. Significant particles have not yet been observed with normal young birds of the sort used in the study of erythromyeloblastic leukosis.

Discussion. There has been demonstrated a particulate material in the plasma of birds inoculated with RPL 12 lymphoid tumor strain. While a variation of approximately 100-fold in the number of particles was found in the group of animals with advanced disease, the occurrence in some instances of large amounts of the material, as in donors M 1305 A (Fig. 2), M 130 Q_2 and the 2 specimens from M 1415 D (Table I), is strongly suggestive of a specific relationship of the particles with the lymphomatous process. These findings are closely similar to those with erythromyeloblastic leukosis in which there is now little doubt that the plasma particles rep-

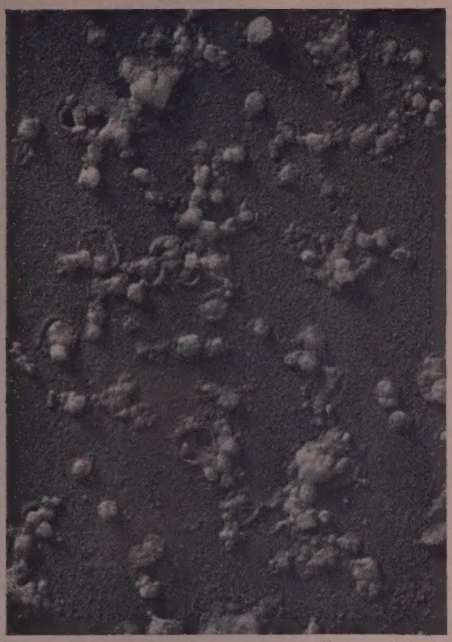

FIG. 2. Electron micrograph of the particles of the concentrate obtained from plasma M 1305 A (see Table I). Magnification 24000 ×.

resent the specific viral agent of that disease. There seems no reason for evading, at least tentatively, the interpretation of the particles in the present work as the virus of one form of lymphomatosis. The diversity of the findings with the individual plasmas is not difficult to explain; variation in the numbers of virus particles is considerable in erythromyeloblastic leukosis in which the stage of the disease is readily diagnosed and in which the cells probably yielding the virus circulate in the blood stream. In contrast, the diagnosis of lymphomatosis in live birds and determination of the optimum time for bleeding is very difficult.

It should be emphasized that no criterion is available by means of which a single particle can be regarded in the electron micrographs as specifically significant. Further, it cannot be said that the particle counts of Table I are of high accuracy. Counts of the particles were made from micrographs of which those of Fig. 1 (A) and (B) represent greatly magnified small areas. Inspection shows that the selection of the particles counted is a matter of judgment based on the resemblance of the one to the many. The counts recorded in Table I indicate, essentially, the minimum number of characteristic particles present.

In the period of about 7 years preceding the present work, during part of which time the tumor material was kept in storage at —76°C, the disease studied here has been passed 12 times in White Leghorn chickens of 1 to 3 days of age by means of plasma, serum or extracts of lymphomatous liver tissue from which the cell material was removed by filtration or centrifugation at 4,000 X g for 40 minutes in the cold. During this time, the disease process has varied somewhat from its original character. Until recently, the primary pathological change was the formation of lymphoid tumors in the viscera, especially the liver, which were typical of those occurring naturally in visceral lymphomatosis. The tumor cells were located extravascularly and accumulations resulted in tumor areas varying in size from those containing only a few dozen cells to others of several cm in diameter. In the last two passages of this tumor strain, most of the deaths occurred in a much shorter time than in previous passages; there was only a moderate enlargement of visceral organs and the same malignant lymphoid cells were confined almost entirely to the vascular networks. Thus, the changes in the liver resemble that found in leukemia though a true leukemic condition in the peripheral blood was seldom seen. It cannot be said at present whether this change is due to an alteration of the virus, an expression of a latent virus or merely an alteration in the reaction of the host. Nevertheless, this form of leukosis is wholly different in its manifestations from the erythromyeloblastic disease studied in this laboratory, and in no instance was there evidence of erythromyeloblastic leukosis in the donors of the plasmas studied here.

If the particles seen in these studies on lymphomatosis are actually the virus responsible for the disease, it would seem that a beginning has been made in the elucidation of the problem of the relations between the virus strains or types causing the various forms of the avian leukosis complex. It is evident that these particles are of the same characters with respect to size, shape and general appearance

as those of the virus of erythromyeloblastic leukosis.

Summary. A particulate material has been obtained from the plasma of chickens affected with the RPL 12 lymphoid tumor strain. Electron micrographs of the particles sedimented directly from plasma and of those in ultracentrifugal concentrates reveal spherical or spheroidal particles of sizes varying from 70 to 160 mμ in diameter and an average diameter of about 117 mμ. Particle counts on the individual plasmas of birds with advanced disease gave values of 1 x 10^7 to 160 x 10^7 particles per ml in contrast with the normal plasmas in which only occasional or no particles of this size range were observed. The findings with this form of the leukosis complex are remarkably similar to the results of analogous studies on erythromyeloblastic leukosis. The particles seen in the present work are interpreted, tentatively, as representing the virus of one form of avian lymphomatosis.

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Serum Precipitable and Butanol Extractable Iodine of Bovine Sera. (19323)

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Dairy and beef cattle differ widely in their ability to secrete milk. Reece and Turner(1) have reported that the thyrotrophic content of the anterior lobe of the pituitary gland of cattle is greater than that in beef cattle. Throughout lactation objective measures of thyroid activity have not been satisfactory. Ruminants, especially lactating ones, are not good subjects for the determination of basal metabolic rates because rarely are they in a post-absorptive state. B.M.R., therefore, cannot be used as a measure of thyroid activity in these animals. Reports in the literature indicate a good relationship between thyroid activity and the serum precipitable iodine (SPI) of men and women(2). In this investigation an attempt was made to study thyroid activity of beef and dairy cattle by comparing sera of 20 dairy cows with sera of 20 beef cows for the content of iodine precipitated with protein. After these 40 sera had been analyzed for SPI the butanol extractable iodines (BEI)(3) of 20 sera were compared with the SPIs of the same sera. The BEI is thought to contain the iodine of "thyroxine-like" compounds(3,4). Comparison of SPI and BEI of men and women had shown that ingestion of inorganic iodine might elevate the iodine precipitated with protein above the "thyroxine-like" iodine compounds by as much as 10 μg %. The BEI correlated more closely than the SPI with the clinical state of patients(3).

Methods. Sera from venous blood were maintained at about 40°F for 1 to 4 days before analysis. SPIs of the first 20 samples were measured by a permanganate acid ashing technic(5,6). Subsequent SPIs were determined by the micro modification of this technic, which requires only 1 ml of serum for each of the duplicate aliquots(2). The BEI(3) values as well as the SPI measurements are the average of duplicate analyses.

Results. The BEIs of nonpregnant, lactat-

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TABLE I. A Comparison of SPI and BEI Determinations of Bovine Sera.

Breed	Animal No.	Age Yr	Age Mo	Days in milk	Iodine, μg % SPI	Iodine, μg % BEI	Iodine, μg % SPI–BEI
Jersey	J23	3	2	50	5.1	3.1	2
	20	3	5	121	5.6	3.8	1.8
	30	4	5	95	3.6	1.6	2
	38	5	4	121	5	2.8	2.2
	26	6	5	59	3.8	1.3	2.5
Avg		4	7	89	4.6	2.5	2.1
Brown Swiss	675	3	5	26	3.7	3.1	.6
	679	3	8	116	3.4	2.2	1.2
	673	4	1	116	4	2.6	1.4
	669	4	9	106	4.2	2.8	1.4
	653	6	10	55	3.1	1.8	1.3
Avg		4	7	84	3.7	2.5	1.2
		Days					
Jersey	J73	28			11.9	3.7	8.2
	69	106			4.7	2.1	2.6
	70	102			4.6	1.5	3.1
	61	302			4.3	3	1.3
	60	371			4.2	2.9	1.3
Avg		182				2.6	
Brown Swiss	693	18			4.5	2.7	1.8
	692	47			7.2	4.6	2.6
	691	59			8.7	4.6	4.1
	689	276			4.9	3.8	1.1
	688	366			4.5	3.8	.7
Avg		153				3.9	

ing Jerseys and Brown Swiss were nearly identical (Table I). The BEIs of Jerseys varied between 1.3 and 3.8 with an average value of 2.5 μg %. The BEIs of Brown Swiss in the same stage of lactation were between 1.8 and 3.1 with an average value of 2.5 μg %. No explanation is apparent why the SPIs of Jerseys were somewhat higher than those of Brown Swiss. The SPIs of the Jerseys were between 3.6 and 5.6 with an average of 4.6 μg %, while the SPIs of the Brown Swiss were between 3.1 and 4.2 with an average of 3.7 μg %. It is difficult to attach any significance to this difference since the average BEIs of the 2 breeds of dairy cattle were identical and since SPI may contain noncalorigenic organic iodine compounds, such as diiodotyrosine.

The average BEI of 2.6 of the Jersey heifers was similar to that of 2.5 μg % of the mature Jerseys. The range of values was also similar. The Brown Swiss heifers, however, which do not mature so rapidly as Jersey heifers, had higher BEI (average 3.9 μg %) than mature Brown Swiss with an average BEI of 2.5 μg %. This difference between the average BEIs of heifers and of mature Brown Swiss is significant because the standard deviation of the duplicates from the averages of 30 determinations was calculated to be 0.28 μg %. The range of SPI for the immature animals varied so widely that no comparison between the two breeds of dairy cattle is justified.

In our first investigation of bovine SPI a comparison was made of Jersey and Aberdeen Angus sera. Four such comparisons were made, each comparison consisting of sera from 5 Jerseys and 5 Aberdeen Angus with the animals in approximately the same stage of lactation or pregnancy. The SPI values for Jersey and Aberdeen Angus sera are recorded in Table II. In 3 of the 4 comparisons the average SPI of the Jerseys was higher than the SPI of the Aberdeen Angus by 1.8, 1.1, and 0.9 μg %. These differences do not indicate greater thyroid activity in Jerseys than in Aberdeen Angus unless the amount which SPI exceeds BEI of Aberdeen Angus is the same as that of the Jersey cow. If the non "thyroxine-like" portion of the SPI differs in the Jersey and Aberdeen Angus, as it did in the Jersey and Brown Swiss, 2.1 and

TABLE II. A Comparison of SPI in Jersey and Aberdeen Angus Sera. 5 animals in each series.

Breed	Age Yr	Age Mo	Days in milk	Days pregnant	SPI, μg % 1	2	3	4	5	Avg
AA*	4	4	50	0	2.6	2.7	2.8	4	4.2	3.3
J†	4	7	72	0	3.7	4.5	5.2	5.6	6.7	5.1
AA	3	0	Dry	216	4.2	4.9	4.9	5.5	5.6	5
J	2	7	Dry	206	4	4.1	4.2	4.3	4.9	4.3
AA	4	4	71	0	4.1	4.3	4.5	4.9	5.1	4.6
J	4	2	51	0	5.2	5.3	5.5	6	6.3	5.7
AA	3	10	Dry	213	3.3	4	4.1	4.2	4.3	4
J	3	10	Almost dry	207	4.4	4.6	4.8	5.2	5.6	4.9

* AA = Aberdeen Angus. † J = Jersey.

1.2 μg %, the differences between the Jersey and Aberdeen Angus BEI may be negligible. A study is now being planned of SPI and BEI of Jersey and Aberdeen Angus sera.

Discussion. The average amount, 1.6 μg %, by which the SPI of 5 nonpregnant, lactating Jerseys exceed the BEI is greater than the average difference, 0.5 μg %, by which the SPI exceeds the BEI of men and women who are not receiving inorganic iodide. This greater difference for cattle may be the result of the iodide contained in the salt blocks available to the dairy animals. For this reason variations in SPI of cattle receiving iodized salt may not be indicative of differences in thyroid activity unless the averages of SPI vary by more than 1.6 μg %, the average difference between SPI and BEI of mature animals. The greater differences between SPI and BEI in young calves may be the result of greater differences in the ingestion of iodized salt.

Summary. Four comparisons (10 sera in each comparison) were made of the SPI content of Jersey and Aberdeen Angus sera. The average SPI of the Jersey sera was 5.0 μg %, whereas that of the Aberdeen Angus sera was 4.2 μg %. In a comparison of Jersey and Brown Swiss sera the former was found to be higher in PBI than the latter, 4.6 and 3.7 μg % respectively; the average BEI of the sera, however, was identical (2.5 μg %). The range of SPI for Jersey and Brown Swiss heifers was so great that no comparison is justified; the average BEI was 2.5 μg % and 3.9 μg % respectively.

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Effect of BAL on Survival of Rats after Lethal Doses of Polonium.*† (19324)

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Earlier experiments(1) have shown that BAL (2,3-dimercaptopropanol) administered intramuscularly after single intravenous doses of polonium doubles the 10-day total excretion

* This paper is based on work performed under contract with the U. S. Atomic Energy Commission at The University of Rochester Atomic Energy Project, Rochester, N. Y.

† This paper is based on University of Rochester Atomic Energy Project Report UR-83.

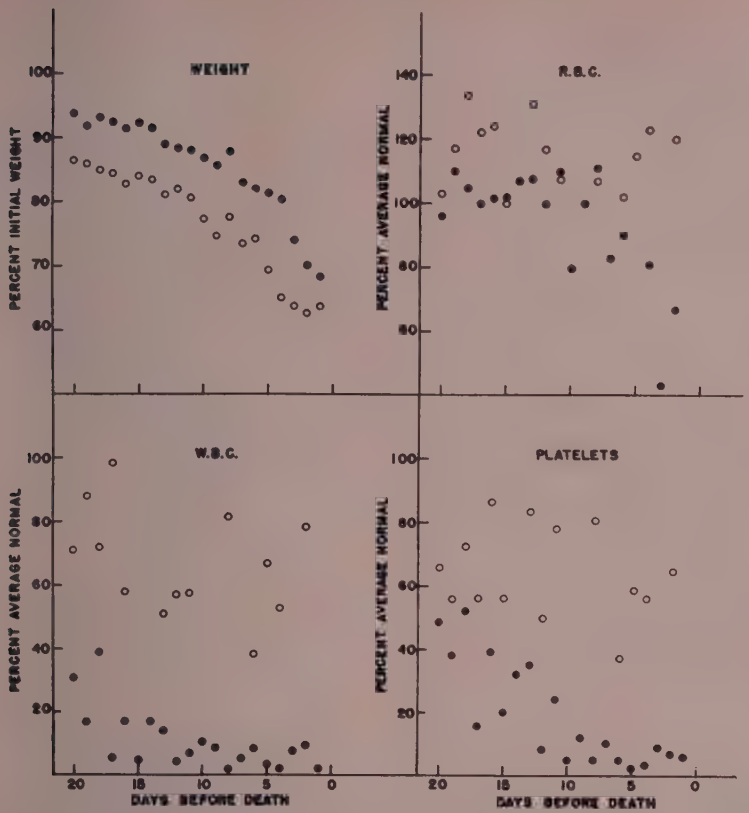

FIG. 1. Average loss of weight and average changes in formed elements of circulating blood are plotted for 20 days preceding death. The dots refer to the control group of rats, open circles to the BAL-treated group.

and brings about a shift of polonium from the bone marrow, spleen, and testis into muscle tissue. On the basis of both these effects it was believed that BAL would significantly modify the toxicity of polonium and the following experiments were performed to test that belief.

Methods. Eighteen male, Wistar, albino rats averaging about 250 g in weight were injected with a polonium dose of 36 μc/kg introduced into the caudal vein of the unanesthetized rat. Immediately thereafter 9 rats received an intramuscular injection of 0.04 ml of 10% BAL in peanut oil, followed by subsequent dosage at the rate of 3 doses per day, 3 hours apart for 3 days. Each rat was weighed daily and clinical signs were noted in the record. Blood counts were made twice a week for the first 40 days, once a week for the next 40 days, and once every 2 weeks for the next 58 days.

Results. The survival times in days as found for the 9 BAL-treated and 9 control rats were as follows:

BAL-treated:	37, 57, 58, 75, (89), 103, 174, 219, 250
Control:	18, 19, 20, 20, (22), 23, 26, 29, 32

Fig. 1 presents the average weight in per cent of initial weight, the average per cent normal red blood count, white blood count, and platelet count for the 20 days immediately preceding death. Since each rat was weighed daily, each point on the weight curve is the average of nine values. Since the blood sampling was staggered, the average value plotted for a single day is based on data from 2 to 4 rats.

The average normal values used to calculate the data in Fig. 1 are derived from an unpub-

lished study of 116 adult male rats of the Wistar strain yielding per cu mm blood: for red blood cells, 8.55 millions (range 7-11); for white cells, 18.82 thousands (range 6-30); for platelets, 8.0 hundred thousand (range 5-10).

The clinical signs recorded for the rats dying in the early stages (the control rats) presented the typical radiation injury syndrome, *i.e.*, lassitude, rough coat, bloody nose, loss of appetite. The BAL-treated animals which died at longer times displayed lassitude and loss of weight without the hematological signs.

Discussion. The median survival time of the untreated rats injected with a polonium dose of 36 μc/kg was 22 days. This compares with a figure of 25 days taken from a curve relating survival time and dose of polonium published by Fink and others(2). The corresponding median survival time for the BAL-treated group was 89 days, which is equivalent to a dose of 17.8 μc/kg based on the Fink curve.

It may be shown that the increased polonium excretion promoted by BAL does not completely account for the prolongation of survival time. On the basis of excretion data published earlier(1) it is known that at the end of 10 days, BAL-treated rats retained 46.5% of the dose and untreated rats retained 74.3% of the dose. After 10 days excretion is not significantly affected by BAL treatment. Therefore, taking account only of excretion we might predict that the treated group would behave as if the effective dose were no less than $46.5/74.3 \times 36 = 22.4$ μc/kg which is equivalent to a median survival time of 57 days on the basis of the Fink curve.

The experimental finding of 89 days median survival time suggests the conclusion that the detoxifying effect of BAL cannot depend only upon increased excretion but must also consist in a mobilization of polonium from radiation-sensitive tissue such as bone marrow and spleen into radiation-resistant muscle tissue.

The effect of BAL is reflected in the contrasted hematology of the treated versus the untreated rat groups just prior to death. Fig. 1 shows that the untreated animals suffer precipitous falls in red and white cell, as well as platelet, counts. Damage to the hemopoietic system was clearly a major cause of death in this group. On the other hand, while the average white cell and platelet counts of the treated animals were reduced somewhat from average normal values, the reduced levels were well maintained and probably presented no serious functional handicap. Beyond the loss of weight preceding death, a common late effect of lethal radiation, the experiment provides no information as to the cause of death for the treated group.

Summary. (1) Control rats injected with a lethal dose of polonium (36 μc/kg) had a median survival time of 22 days, whereas the BAL-treated group had a median survival time of 89 days. (2) Blood studies of control rats showed the typical picture of hemopoietic failure in contrast with well sustained cell and platelet levels found for the BAL-treated group. The difference is believed to be due to a reduced radiation exposure of the spleen and marrow caused by the action of BAL in accelerating excretion and in diverting polonium from the hemopoietic organs into muscle.

The author wishes to thank Mary Elizabeth Hudson and Ann Marie Noonan for making the considerable number of blood counts necessary to carry out this experiment.

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Effect of Ultraviolet on Electrical Properties of Nerve.* (19325)

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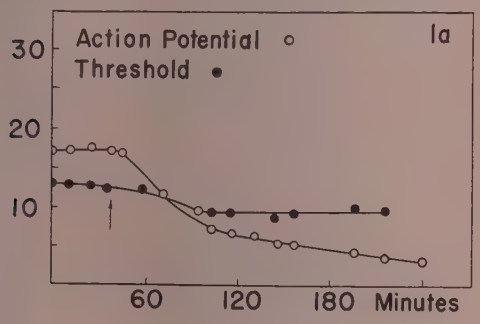
We investigated the effect of a single wave length (2537A) on the action and resting (injury) potentials, the threshold and the recovery changes following a single shock.

Experimental. The sciatic nerve of *Rana pipiens* dissected to the ankle was kept immersed in Ringer's solution on polished silver electrodes during irradiation. The action potential was measured with a differential amplifier feeding into an oscilloscope. Square waves of 0.1 millisecond duration were delivered to the nerve at a frequency of 3/sec. Injury potentials were measured with a Leeds and Northrup type K potentiometer and a Rubicon 3402HH galvanometer. Electrodes were calomel half-cells led to the nerve via agar-KCl bridges. Measurements of excitability changes were made by noting the strength of a second stimulus required to give 50% of the total A fiber response at given intervals following a maximal conditioning A shock. The ultraviolet source was a General Electric sterilamp giving more than 85% of its ultraviolet output at 2537A. The intensity of the irradiation was of the order of 100 egs/mm^2/sec. and was continuous throughout each experiment.



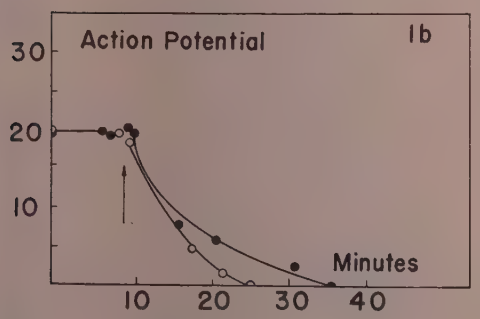
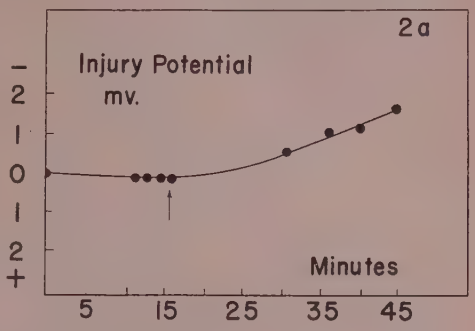


FIG. 1a. Effect of ultraviolet on action potential and threshold of frog sciatic nerve. The arrow indicates the onset of radiation. Fig. 1b. Effect of ultraviolet on action potential of the peroneal branch of the frog sciatic nerve. Intensity for lower nerve (circles) 2.4 times that of upper (dots).



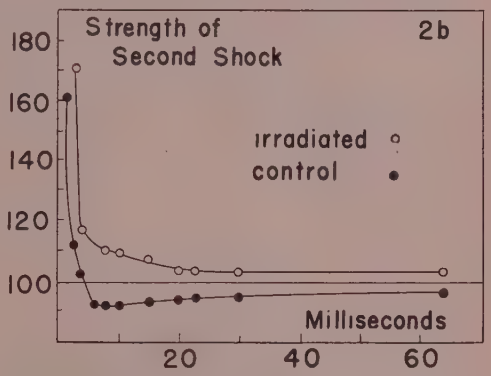


FIG. 2a. Depolarizing effect of ultraviolet on the frog sciatic nerve. Fig. 2b. Effect of ultraviolet on excitability changes following a single shock.

Results. Intense irradiation typically produced a sigmoid shaped survival curve (Fig. 1a). The survival time of a thin nerve as

* Supported in part by a grant from the University of Kentucky Research Fund.

illustrated in Fig. 1b was only 15 minutes. Survival times for the sciatic nerve ranged from 1.5 to more than 4 hours over all seasons (11 experiments). An increase of intensity decreased the survival time (Fig. 1b). If failure is exclusively the result of the lowering of the action potential below a critical level, little change in threshold would be expected. A decrease in threshold averaging 15% in 10 experiments (Fig. 1a) was found. The extreme rise obtained with single fibers(1) was absent. Continuous irradiation leads to a progressive depolarization of the treated region. Six identical results were obtained; one is illustrated in Fig. 2a. During irradiation the supernormal period disappears and the relative refractory period becomes extensive (5 experiments) as shown in Fig. 2b. Cysteine (0.02 M), at pH 7, calcium chloride (0.02 M) and visible light (2000 watts) were ineffective in retarding the fall in action potential. Both short and long periods of illumination were equally ineffective in producing photoreactivation.

Discussion. Ultraviolet appears to affect nerve in much the same way as several metabolic inhibitors. Cyanide and nitrogen, for example, cause a fall in resting potential with little or no change in threshold(2). Ultraviolet differs from the effect of methylfluoracetate(3) in that the latter affects the threshold strikingly while leaving the resting potential unaltered. It appears probable that a major effect of ultraviolet on nerve is the result of the inhibition of one or several respiratory enzymes. Since the magnitude of the threshold change is slight, little direct effect on the membrane seems involved.

Summary. Ultraviolet irradiation of nerve at 2537A leads to a fall in action potential, a depolarization and a prolongation of the relative refractory period. A slight fall in threshold was observed.

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Effect of Hypophysectomy on Pigmentation and Ascorbic Acid Excretion in Black Rats.* (19326)

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It was previously observed that adrenalectomy in black rats was associated with a marked increase in the deposition of melanin in the hair follicles and bulbs in these animals(1). This report is concerned with the effect of hypophysectomy on pigmentation in black rats.

Experimental procedure. Male and female black rats of the Long-Evans strain, bred in our laboratories, were hypophysectomized when the animals were between 50 and 60 days of age, by the Hormone Assay Laboratories in Chicago. The weight of the males ranged from 125 to 150 g and of the females from 100 to 125 g. After hypophysectomy, the rats were fed a normal experimental diet supplemented with small amounts of canned dog food or evaporated milk. The fur on the back was shaved and the degree of pigmentation and hair growth recorded. A number of animals were placed in metabolism cages and the urine was collected for 24-hour periods. Ascorbic acid was determined by the method of Rowe and Kuether(2). At the conclusion of the experiments, the rats were sacrificed and the adrenal glands were removed, weighed, and prepared for pathological study.

* This research was aided by grants from the Josiah Macy, Jr. Foundation and from the National Vitamin Foundation.

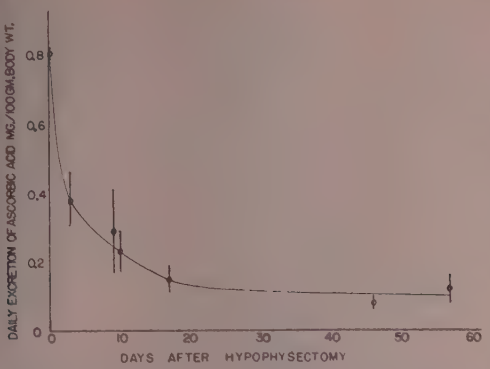

FIG. 1. Daily excretion of ascorbic acid by hypophysectomized rats is in mg of ascorbic acid per 100 g body weight. Vertical lines indicate the standard errors.

Results. Pigmentation. Seven or 8 days after hypophysectomy, a bluish pigmentation was visible through the thin overlying cutis of the black hypophysectomized rats, similar to the pigmentation previously observed in black rats following adrenalectomy(1). The pigmentation was not as uniformly intense as that observed in the adrenalectomized rat, but the deep blue coloration due to increased melanin deposition was clearly visible through the thin overlying cutis. This coloration appeared 7 or 8 days following hypophysectomy and reached the maximum at 15 to 17 days. After 20 days, the pigmentation gradually faded to a grayish color.

Ascorbic acid excretion. The data on ascorbic acid excretion are summarized in Fig. 1. Prior to hypophysectomy, the rats excreted 0.80 ± 0.14 mg of ascorbic acid per 100 g of body weight in 24 hours. Following hypophysectomy, the ascorbic acid excretion decreased, reaching a level of about 0.1 mg per 100 g of body weight by 50 days after hypophysectomy. We previously reported that adrenalectomized rats on identical diets excrete approximately 0.25 mg of ascorbic acid per 100 g of body weight(3).

Adrenal weights. The mean weights of the adrenals of normal male and female rats 60 days of age and of hypophysectomized male and female rats are shown in Table I. Without exception, the adrenals of the hypophysectomized rats were found to be smaller than the adrenals of normal 60 day old rats, the age at which the experimental rats were hypophysectomized. Pathological study revealed marked atrophy of the adrenal cortex of the hypophysectomized rats.

Discussion. The development of intense pigmentation in the hair apparatus of black rats following hypophysectomy supports the suggestion previously made that the increased deposition of melanin following adrenalectomy is related to the absence of the steroid hormones of the adrenal cortex(1,4). The observations that pigmentation in adrenalectomized black rats was inhibited by the daily injection of DCA tends to support this suggestion(4). The decrease in the excretion of ascorbic acid which occurred in both adrenalectomized(3) and hypophysectomized rats may be related to the increased deposition of melanin in these animals. It has been reported that the administration of ascorbic acid will correct a disorder in the metabolism of tyrosine and phenyl alanine that occurs in scorbutic guinea pigs(5) and in human infants(6).

Obviously, the decreased excretion of ascorbic acid by hypophysectomized and adrenalectomized rats is only one factor which may contribute to the increased pigmentation in these animals, as the increased pigmentation fades after four weeks, while the decreased excretion of ascorbic acid continues throughout the life of the animal.

Summary. Pigmentation was observed in

TABLE I.

	No. of rats	Age, days	Avg wt of rats, g ± S. E.	Absolute wt in mg ± S. E.	Wt in mg/100 g body wt ± S. E.
Intact ♂	8	60	129 ± 9	20 ± .8	15.6 ± .8
♀	6	60	110 ± 5	22 ± .4	20.2 ± .8
Hypophysectomized ♂	11	111-185	133 ± 7	8.9 ± .6	6.9 ± .6
♀	6	111-185	112 ± 3	9.3 ± .4	8.3 ± .4

black rats following hypophysectomy, similar to the pigmentation occurring in black rats following adrenalectomy. The adrenals of the hypophysectomized rats were smaller than normal, with atrophic cortices. Excretion of ascorbic acid decreased to low levels following hypophysectomy. The increase in pigmentation is believed to be related to the derangement of the steroid hormones of the adrenal cortex of the hypophysectomized rat.

We are indebted to Dr. Hammond Pride for his assistance in part of these experiments.

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In Vitro Effect of Aureomycin, Terramycin, and Chloramphenicol on Typhus Rickettsiae.* (19327)

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In a previous communication(1) from this laboratory it was reported that purified typhus rickettsiae oxidize glutamate, and that the rate of this oxidation is proportional to the concentration of viable rickettsiae, as determined by their toxicity for white mice. It was further shown(2) that the antibiotics effective in rickettsial infections, aureomycin, terramycin, and chloramphenicol, do not reduce the toxicity for white mice when tested *in vitro* for one hour at concentrations of 10 and 30 μg per ml. The present paper deals with the effect of these antibiotics in concentrations of 10-300 μg per ml on the rate of glutamate oxidation and the toxicity for mice of purified suspensions of epidemic and murine typhus rickettsiae.

Materials and methods. Antibiotics. Aureomycin hydrochloride (twice recrystallized, Lederle), terramycin hydrochloride (Pfizer), and chloramphenicol (synthetic, Parke, Davis)† were used. In some experiments, terramycin sterile base (Pfizer), and chloramphenicol (pure crystalline) were used; however, no differences between the two preparations of these drugs were observed. The antibiotics were dissolved in a salt solution of the following composition: KCl 0.126 M; NaCl 0.0018 M; KH_2PO_4 0.0012 M; Na_2HPO_4 0.0109 M (solution K 7.5)(3); the pH was adjusted to 7.5 by addition of KOH, and dilutions were made with solution K 7.5. *Rickettsial suspensions* (the Wilmington strain of *R. mooseri* and the Breinl strain of *R. prowazeki*) were prepared from frozen infected yolk sac pools as described previously (1,3). The precipitate from the final high speed centrifugation was resuspended in either sucrose-P (sucrose 0.225 M; KH_2PO_4 0.0016 M; K_2HPO_4 0.0086 M, pH 7.5)(3), or 6% bovine serum albumin powder, Armour's fraction V, in solution K 7.5, pH 7.5. In all experiments, 1 ml of the suspending fluid was used for each 2 g of original infected yolk sac. *The rate of oxygen uptake* was measured by the Warburg method at 34.5°C. Flasks of two capacities, 3 ml and 8 ml, both

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smaller than the conventional size, and manometers of appropriate size were used. In all cases the reaction mixture in the 3 ml flasks consisted of 0.25 ml of the 200% rickettsial suspension, 0.05 ml of 0.16 M glutamic acid neutralized with KOH, 0.05 ml of a mixture of 0.024 M $MgCl_2$ and 0.004 M $MnCl_2$, and 0.05 ml of antibiotic solution to give the indicated final concentration. The center well contained 0.05 ml 10% KOH. The 8 ml flasks were employed in those experiments in which the antibiotic was added from the side arm. The reaction mixture in these experiments consisted of 0.75 ml of the 200% rickettsial suspension, 0.1 ml of 0.16 M glutamic acid neutralized with KOH, 0.1 ml of a mixture of 0.024 M $MgCl_2$ and 0.004 M $MnCl_2$, 0.1 ml of solution K 7.5, and 0.15 ml of antibiotic solution. The center well contained 0.05 ml 10% KOH. *Toxicity for white mice* of the contents of the Warburg flasks at the end of the experiment was estimated by the intravenous injection of 0.25 ml of serial half log (3.16 fold) dilutions, using 4 mice for each dilution(4). The dilution required to kill 50% of the mice was calculated by the method of Reed and Muench(5). The mice surviving the toxic action of suspensions of murine rickettsiae were observed for 8 days, and deaths due to rickettsial infection as determined by autopsy were recorded.

Results. The rate of oxygen uptake with glutamate of the rickettsial suspensions used in these experiments ranged from 43 to 90 micro-liters (μl) O_2/hr/ml suspension; the oxygen uptake without added glutamate was negligible. With all three antibiotics at concentrations comparable to those effective *in vivo* (10-30 μg per ml), a slight but definite inhibition of the rate of oxygen uptake with glutamate was observed. This effect has been noted by Wisseman *et al.*(6). Higher levels of aureomycin and terramycin (100-300 μg per ml) caused increasingly stronger inhibition, while higher levels of chloramphenicol were without further effect. Typical experiments showing the progress and degree of inhibition are presented in Fig. 1. When aureomycin or terramycin was added to the respiring rickettsial suspensions, there was an immediate inhibition which increased rapidly for the first 30 minutes. In the case of aureomycin, this increase continued at a slower rate during the remaining 150 minutes. However, in the case of terramycin the levels of inhibition reached in the first 30 minutes remained constant throughout the period of observation. In the experiments presented here murine rickettsiae suspended in the 6% albumin solution were used. When the sucrose solution was used instead of the albumin one, the re-

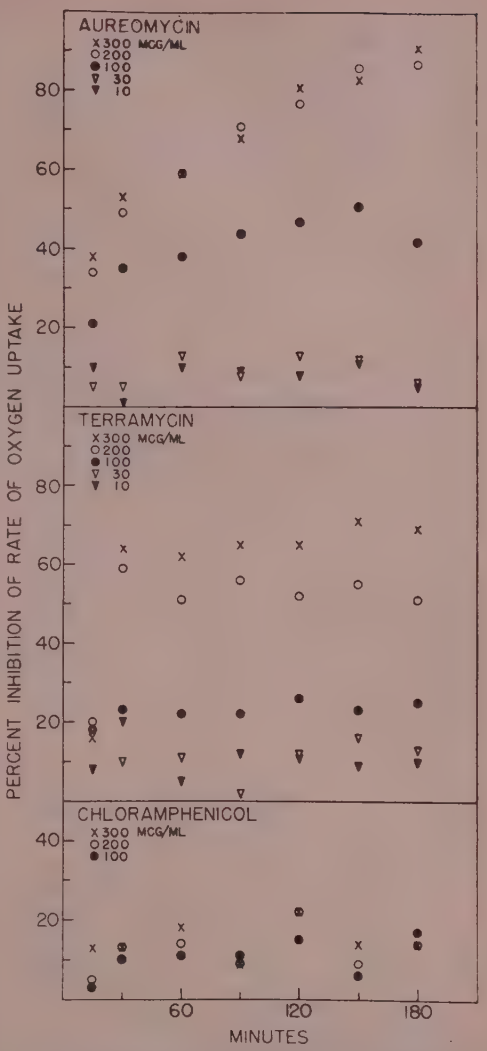

FIG. 1. Effect of aureomycin, terramycin and chloramphenicol on the rate of glutamate oxidation by murine typhus rickettsiae.

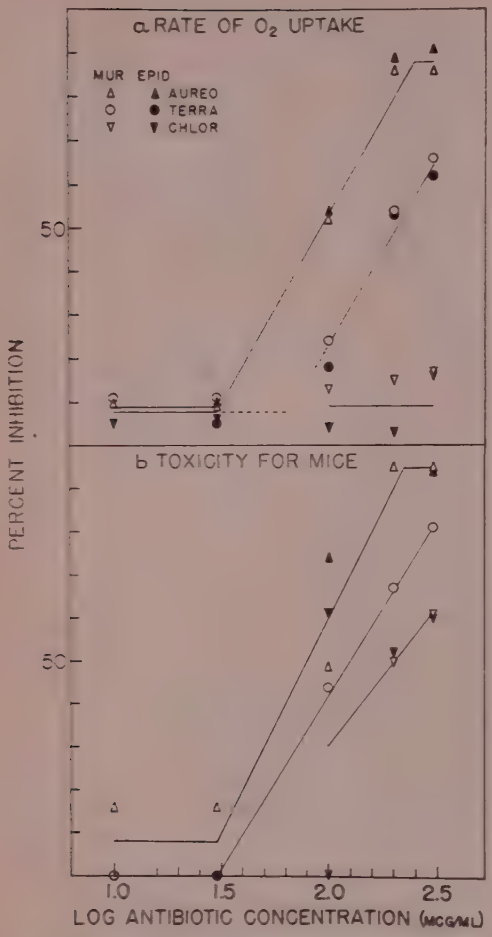

FIG. 2. Extent of inhibition of glutamate oxidation (a) and toxicity for mice (b) of epidemic and murine typhus rickettsiae with different levels of aureomycin, terramycin and chloramphenicol.

sults with aureomycin were essentially the same; with terramycin, although the same degree of inhibition as in albumin was attained (at concentrations of 300, 200, and 100 μg per ml), the progress of the inhibition was much more gradual. Thus, with 300 μg terramycin per ml, 50% inhibition was reached in albumin in about ½ hour, and in sucrose in about 2 hours. The respiration of epidemic typhus rickettsiae in albumin or in sucrose was affected by all three antibiotics in the same way as that of murine rickettsiae.

The relative effectiveness of the antibiotics as inhibitors of glutamate oxidation by both murine and epidemic typhus rickettsiae is shown in Fig. 2a. A linear relationship between the logarithm of the drug concentration and the degree of inhibition may be observed at concentrations above 30 μg per ml for aureomycin and above 70 μg per ml for terramycin. Thus, on a weight basis aureomycin appears to be about twice as effective as terramycin. The slight inhibition at lower levels of these two drugs and at all levels of chloramphenicol up to 300 μg per ml is independent of drug concentration. The inhibition of the toxicity for white mice of the rickettsial suspensions by aureomycin and terramycin paralelled rather closely the inhibition of respiration (Fig. 2b). In the case of chloramphenicol the toxicity for mice was inhibited more strongly than the glutamate oxidation.

The 3 antibiotics suppressed the infectivity of murine rickettsiae for white mice to approximately the same extent as the toxicity. However, the method used here to estimate infectivity is not sufficiently sensitive to allow accurate correlation.

Discussion. The concentration of the rickettsial cells used in the present *in vitro* experiments was approximately 10^4 times as high as in the *in vivo* chick embryo experiments(2). The influence of the concentration of bacterial cells on the bactericidal action of terramycin has been investigated by Hobby *et al.*(7). They showed that with *S. typhosa* and *P. pyocyaneus* the decrease in the number of organisms after contact with the antibiotic varied not only with the concentration of the antibiotic but also with the concentration of cells. The fact that marked inhibition of respiration and of toxicity for mice was observed only at levels considerably higher than those shown to be effective in chick embryos may be due to the much greater concentration of rickettsiae. Moreover, the rickettsial suspensions used here most likely consist of a mixed population of cells of varying susceptibility to the action of poisons. Young cells, *i.e.*, those having undergone recent cell division, are probably most sensitive, and the slight but definite inhibition observed at low levels of antibiotic may be due to the effect of the drugs on these cells. In the chick embryo or experimental animal continuing cell divi-

sion could account for the marked effect of the antibiotics at low levels.

The antibiotics do not act specifically on glutamate oxidation, for in all cases an equal or greater action on the toxicity and infectivity for mice occurred. The earlier finding that glutamate oxidation is intimately associated with viability(1) is here confirmed, and the depression of this oxidation by aureomycin and terramycin is most likely due to their rickettsicidal action.

It is of interest to note that aureomycin, at concentrations corresponding to those used in the present experiments, specifically depressed phosphorylation in mitochondria without affecting their respiration(8). This may suggest the mode of action of the antibiotic to be an interference of energy transfer. Observations by Hahn and Wisseman(9) on the effect of aureomycin, terramycin, and chloramphenicol on the formation of adaptive enzymes by *E. coli* support this hypothesis. Our present lack of knowledge of the energy metabolism and synthetic reactions of rickettsiae precludes speculation on a similar mechanism for the action of these antibiotics on rickettsiae.

Summary. Aureomycin and terramycin in concentrations of 100-300 μg per ml were found to inhibit markedly the respiration of purified murine and epidemic typhus rickettsiae *in vitro*. Chloramphenicol in concentrations up to 300 μg per ml, aureomycin and terramycin in concentrations up to 30 μg per ml produced only slight inhibition. The inhibition of respiration was correlated with a decrease in the number of viable rickettsiae as determined by toxicity and infectivity for white mice. These experiments demonstrate the rickettsicidal action of aureomycin and terramycin *in vitro*.

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Citrovorum Factor, Vitamin B_{12}, and Folic Acid Activity of Whole Blood of Several Species.* (19328)

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In the course of a series of investigations concerning the release of *Leuconostoc citrovorum* factor (LCF) from tissues(1) and the conversion of folic acid (PGA) to LCF(2), we became interested in the LCF content of blood. This paper reports studies on the LCF, vit. B_{12}, and PGA content of the blood of rats (in various stages of vit. B_{12} depletion or repletion), chicks, sheep, and cattle.

Experimental. Blood was obtained from rats under Nembutal† anesthesia by hypo-

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† Pentobarbitol sodium, Abbott.

TABLE I. Free Vitamin Content and Effect of pH of Autolysis on Release of *S. faecalis* and *L. citrovorum* Activity from Whole Blood and Plasma.*

	Mature rat, non fasted					Mature rat, fasted 24 hr					Veal calf				
	CF		PGA		B_{12}	CF		PGA		B_{12}	CF		PGA		B_{12}
pH	Free	Total	Free	Total	Free	Free	Total	Free	Total	Free	Free	Total	Free	Total	Free
4.5		5.2		9			1		3.8			2.6		4.5	
5		11.3					1		3.9			2.5		4.5	
5.5		12.3		20			2		4			2.1		4.5	
6		5.4					2.2		4.2			2.4		5.5	
6.5		7.8		10			2.3		3.5			2.4		6	
7	<.2	11.6	2	19.5	.70	<.2	3.2	3.3	4.3	.50	<.1	1	.5	9	.97
7.5		8.2		12			2.8		4.2			.9			
8		6		12			1.8		2.8						

	Chicken					Sheep					Ox plasma				
4.5		3		<4			.1					2.9		6.5	
5		7.5		19			.9		1.5			2.1		7.5	
5.5		16.2		23.1			1.1		2.5						
6		18		37.5			1.2		3.5			3		4	
6.5		16.5		51.5			1.2		4.2			1.3		5.5	
7	<2	13.5	6.1	21	1.25	<.2	1.4	.50	6	.80		1.8		8.5	
7.5							.5		5.4			1.6		3.5	
8		4		12			.5								

* All values are mγ/ml.

dermic syringe from the portal vein, or from the post cava after the rat had been stunned by a blow on the head. The blood from the chick, sheep, and ox was obtained at slaughter. In all cases blood was collected into a chilled container containing double oxalate(3). For the determination of the free vitamin, 1 ml of blood was diluted with 5 ml water, adjusted to pH 7 and autoclaved for 10 minutes at 120°C. The resulting precipitate was homogenized and 4 ml of water was used to rinse the homogenizer. The particulate matter was centrifuged down and the supernatant liquid poured off and frozen at –4°C until ready for analysis.

In the study of the release of bound LCF and PGA, samples of blood were placed in appropriate buffers (citrate-phosphate, or bicarbonate) and incubated at 37°C, under toluene, for 20 hours. After incubation they were adjusted to pH 7, autoclaved for 10 minutes at 120°C, homogenized, and centrifuged. The supernatant solution was removed, and frozen until ready for analysis. Samples for the determination of vit. B_{12} activity were incubated with trypsin (50 mg Difco trypsin ml of heated blood) for 18 hours and treated as outlined above. The vitamin content of the samples was determined by microbiological assay. When large amounts of blood were available, turbidimetric assays with an 18-22-hour incubation at 37°C were employed. When the amount of sample was limited as in the case of individual rats, 72-hour acidimetric assays were run. Vit. B_{12} was determined with the medium of Thompson *et al.*(4) using *Lactobacillus leichmannii* 4797 as the test organism. Total *Streptococcus faecalis* R activity was determined with the medium of Luckey *et al.*(5) and *Leuconostoc citrovorum* 8081 activity was determined using the procedure of Sauberlich and Baumann(6).

Results and discussion. It was found that concentrations greater than 1 ml of blood in 5 ml total volume interfered with the assay of LCF and PGA regardless of whether acidimetric or turbidimetric procedures were used. Therefore, the reported values are for 1 ml of blood in 10 or more ml total volume. In a few cases a 1:20 or 1:30 dilution interfered with the turbidimetric assay. However, such cases were the exception and occurred only with the turbidimetric assay.

Whole blood was found to contain low levels of free LCF and PGA as is shown in Table I. A considerable portion of the vit. B_{12} activity was free or else was released by hemolysis and autoclaving. However, treatment with trypsin at pH 8.2 increased the level of measurable B_{12} slightly. The PGA values are in fair agreement with those reported by Simpson and Schweigert(7). The

TABLE II. The Effect of Vit. B_{12} on the Level of B_{12}, LCF and PGA in the Blood of Vit. B_{12} Deficient Hyperthyroid Rats.

Treatment	Vitamin activity, mγ/ml whole blood		
	LCF	PGA	B_{12}
Control (1)*	.10	14	.30
" (pooled 3)	.10	14	.21
3 γ vit. B_{12} inj./day (1)	2	14	1.50
" " " " " (pooled 3)	2	10	1.25
" " " oral/day (1)	2.9	11	.90
" " " " " (pooled 3)	2.8	14	1.00

LCF and PGA activity determined after autolysis at 37°C, pH 7 for 18 hr. B_{12} activity determined after incubation of 1 ml heated blood with 50 mg trypsin for 24 hr at pH 8.2. There was no free vit. B_{12} in the blood of group III.

* Figures in parentheses represent number of animals used.

vit. B_{12} values were in the range of those reported by Couch *et al.*(8).

The amount of LCF and PGA activity released by autolysis varied with the pH of the reaction medium (Table I). The optimum pH for the release of LCF and PGA by whole blood enzymes was 7.0 and 7.0 for the rat, 4.5 and 6.5 for the calf, 6.0 and 6.5 for the chicken and 7.0 and 7.0 for the sheep. Ox plasma appeared to have 2 points of release at pH 4.5 and 6.0 for LCF and 4.5 and 7.0 for PGA. Simpson and Schweigert(7) reported an optimum pH of 6.0 for the conjugase of the whole blood of rabbits, of 8.0 for turkey blood and 7.0 for turkey plasma. These data suggest that there are at least 3 factors active in the release of PGA and LCF activity from blood. Simpson and Schweigert (7) reported a large increase of PGA activity following treatment with chick pancreas. Hence, the values reported here may not represent total activity present, but merely that released by blood enzymes.

The blood of rats on a vit. B_{12} deficient diet containing 0.15% iodinated casein was very low in LCF (Table II). Blood from similar rats receiving 3 μg of vit. B_{12} per day, orally or by injection, contained appreciable LCF. There was no detectable change in the PGA activity upon the administration of vit. B_{12}. The B_{12} level increased significantly following vit. B_{12} administration.

Summary. (1) The whole blood of the rat, chick, ox, or sheep was found to contain little or no free *L. citrovorum* active material. However, demonstrable amounts of free *S. faecalis* activity were found. (2) The release of LCF and PGA during autolysis is dependent on the pH of the reaction mixture. Optimum values for LCF and PGA were obtained at pH 4.5 or 6.5-7.0 depending on the species involved. (3) Blood from rats on a B_{12} deficient diet containing iodinated casein had little LCF activity. However, blood from hyperthyroid rats receiving ample vit. B_{12} contained LCF levels similar to those of normal rats after fasting.

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Distribution of Mumps Virus in Tissue Cultures as Determined by Fluorescein-Labeled Antiserum.*† (19329)

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Tissue cultures make possible the study of viruses under conditions which are simpler and easier to control than those prevailing in the intact animal or embryo. The detection of viruses in tissue cultures, on the other hand, necessitates the use of cumbersome and expensive procedures which, in the final analysis, yield only indirect evidence as to what is actually taking place in the cells. This is particularly true of viruses such as mumps which do not form inclusion bodies or cause any visible tissue damage. The development of fluorescent antibody(1), and its successful application to the microscopic localization of mumps virus antigen in the parotid glands and central nervous system of experimentally infected monkeys(2,3), opened a new approach to numerous problems relating to the growth of viruses in tissue cultures.

The present experiments deal with the use of the method in a study of mumps virus in various kinds of chick embryonic tissue infected *in vitro*. The answers sought were: 1) Is the amount of virus produced in tissue cultures sufficient to be detected by the fluorescein labeled antiserum; 2) what is the distribution of the virus in the infected tissues; 3) is it the same or different with different kinds of tissue?

Materials and methods. Tissue cultures of the Maitland type were prepared in a manner similar to that described by Weller and Enders (4). The tissues used were the amniotic and chorioallantoic membranes, whole embryo, and brain of 7-8-day-old chick embryos. The tissues were minced with scissors and centrifuged in graduated test tubes at 1,000 rpm for 2 minutes. The fragments were resuspended with medium, consisting of 1 part ox serum ultrafiltrate‡ to 2 parts of Hanks' balanced salt solution(5) and made up to a 10% suspension by volume. The tissue cultures were set up in 50 ml Erlenmeyer flasks fitted with tight rubber stoppers. The volumes used were 0.5 ml of the 10% tissue suspensions, 3.5 ml of medium and 0.1 ml of a 10^{-2} dilution of the stock virus. The latter consisted of pooled allantoic fluid derived from the 62nd egg passage of the Enders strain of mumps. Duplicate cultures were prepared with each of the tissues and incubated at 35°C. The controls included: 1) Uninoculated cultures; 2) cultures in which the tissues were killed by freezing prior to inoculation; and 3) inoculated cultures held at 4°C. After 7 or 14 days of incubation, duplicate cultures were pooled and centrifuged. The supernatant fluids were pipetted off, tested for the presence of hemagglutinins and inoculated intra-amniotically in 0.1 ml amounts of serial 10-fold dilutions, into groups of 8, 7-8-day-old chick embryos to determine their infectivity. The eggs were incubated for 7 days and tested for the presence of hemagglutinins according to the methods previously described(4,6). The sedimented tissues were resuspended in 1 ml amounts of a 10% solution of gelatin, transferred from the graduated centrifuge tubes to ordinary bacteriological test tubes and placed in the incubator. The tubes were shaken occasionally to insure the penetration of the gelatin around the tissue fragments and after about 1½ hours, centrifuged and chilled in an ice water bath to solidify the gelatin. The gelatin-imbedded tissues were loosened from the tubes by dipping them into hot water, shaken out into chilled Petri dishes and cut into appropriate blocks. The blocks were returned to the respective tubes, being placed on the wall about half way down the tube. The tubes were closed with rubber stoppers and the tissues quick-frozen in an alcohol dry ice mixture and stored at –20°C.

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† Aided by a grant from the National Foundation for Infantile Paralysis.

‡ Purchased from Microbiological Associates, Bethesda, Md.

FIG. 1. Surface staining of piece of brain tissue from Maitland type of tissue culture 14 days after inoculation. × 84.

FIG. 2. Staining of cyst-like structures in piece of chorioallantoic membrane from Maitland type of tissue culture 14 days after inoculation. × 84.

FIG. 3. Section of chorioallantoic membrane showing 2 cells in which entire cytoplasm is stained and 2 in which brightly fluorescent granules are arranged around a centrally unstained nuclear shadow. × 360.

The preparation of the frozen sections, the procedure used in their staining and the controls of specificity were carried out according to the methods described by Coons *et al.* (1,7). The fluorescein conjugated antimumps serum used in this experiment was also prepared in the same way and from the same batch of convalescent monkey serum as that employed in (2) and (3) with the exception that it was absorbed twice with chicken liver powder instead of human or mouse liver powder prior to its use as a stain.

Results. Areas of bright yellow-green fluorescent staining were seen only in sections prepared from cultures with living tissues incubated at 35°C. This staining occurred in most though not in all the pieces of tissue examined on a given slide. The specific fluorescent precipitate was deposited over the cells lying at the extreme periphery of the larger tissue fragments but not in the central regions. The stained cells were distributed individually or in clusters in isolated or irregularly scattered patches at the edges of the sections. Occasionally they formed a halo-like ring around the entire edge of the fragment (Fig. 1). Some of the smaller pieces of tissue, which were usually no larger than 3-4 cell layers thick, were stained throughout. Specifically stained cellular debris and granular material were also present. The thin-walled cyst-like structures seen in many of the pieces of amniotic and chorioallantoic membranes (which according to Weller and Enders (4) represent areas of active tissue growth) were frequently outlined by the presence of the fluorescent precipitate (Fig. 2). With this exception very little difference could be detected in the distribution of the virus in cultures with the various kinds of tissue examined. Thus, whether the tissue was amnion, chorioallantois, whole embryo, or brain, the fluorescent precipitate was deposited in an irregular fashion over the surfaces which were directly exposed to the inoculum.

Within the stained cell the detectable virus antigen was found to be located in the cytoplasm but not in the nucleus (Fig. 3). In some of the cells the entire cytoplasm was clearly outlined by the specific staining. In others brightly fluorescent granules of various sizes were seen. These were often arranged in a circular or semicircular fashion around the central unstained nuclear shadow. Due to the embryonic nature of the cultures the identity of the cells which reacted with the conjugate could not be determined. The formation of the fluorescent precipitate was specifically inhibited by preliminary treatment of the sections with unlabeled immune serum.

The multiplication of the virus was established by the appearance of hemagglutinins and by an increase in the infectivity of the culture fluids. However, whereas all the 7-day culture fluids were infective through the highest dilution tested (10^{-6}), the titer of hemagglutinin was 1:16, 1:8, 1:4 and 0 respectively

in the amniotic, chorioallantoic, whole embryo and brain cultures.

No virus could be demonstrated in either the tissue or the supernatant fluid of the uninoculated cultures or of the cultures in which the tissues were frozen prior to inoculation. When infected cultures were stored at 4°C, however, the virus survived through a 10^{-2} dilution of the fluid but could not be demonstrated in the cells.

Discussion. Specific fluorescent immune serum has been used successfully as a histochemical tool in several fields of investigation (1-3, 7-10). Its application to problems dealing with the growth of viruses in tissue cultures has been suggested(11). In the present studies it has been shown that the amount of mumps virus produced in tissue cultures of the Maitland type is sufficient to be detected by fluorescein labeled antiserum. The distribution of the virus was found to be restricted to the cells which came in direct contact with the inoculum irrespective of the kind of tissue used. This would indicate that to increase the yield of the virus in a given culture it would be necessary to increase not the total amount of tissue but its surface area. Since the staining seen in cultures with amnion, chorioallantois, whole embryo mince and brain tissue did not differ significantly, the results suggested that under the conditions used the cells which supported the growth of this agent were either widely distributed or that the virus had no predilection for any particular type of cell. The occasional gaps seen in the peripheral staining of the individual tissue fragments were believed to be attributable to non-viable cells. The presence of the staining in the actively proliferating cyst-like structures seen in cultures of amniotic and chorioallantoic membrane indicated that the virus was capable of infecting cells which had multiplied *in vitro*. Because of the embryonic nature of the cultures, the identity of the cells which precipitated the conjugate could not be determined.

Summary. Fluorescein-labeled antibody has been successfully employed in the localization of mumps virus in cultures of various types of chick embryonic tissues infected *in vitro*. The distribution of the virus was found to be restricted to the cytoplasm of the cells which came in direct contact with the inoculum irrespective of the kind of tissue used in the culture.

The technical assistance of Jeanne M. Connolly is gratefully acknowledged.

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Rapid Determination of Plasma Tocopherols.*† (19330)

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Since present methods for the chemical determination of blood tocopherols (Vit. E) are inconvenient for routine use, we wish to report that adaptation of the micro method of Quaife and coworkers(1) to a macro scale affords a simple and rapid technic suitable for this purpose. The micro method is excellent where only small amounts of blood are available, but the procedure is time-consuming and requires special equipment and technics. As a macro method, using 1 to 3 ml of serum or plasma, the determination requires only routine clinical laboratory apparatus and technics, and approximately twice as many samples may be run in a single working day. We therefore believe that publication of experimental details will be of assistance to those investigators whose research requires study of Vitamin E in blood or other biological materials.

Method. The procedure follows that of the micro method, involving: (a) Deproteinization of the sample with an equal volume of ethanol; (b) extraction with 5 ml of xylene; (c) estimation of carotene color intensity in a 3 ml aliquot of the xylene extract; (d) and reaction of the extract with 1 ml of ferric chloride (1.2 mg/ml in absolute ethanol) and 3 ml of α, α'-dipyridyl (1.0 mg/ml in *n*-propanol). The extraction is performed in glass-stoppered test tubes shaken mechanically for 10 minutes, and the optical densities are determined in an Evelyn colorimeter, using a 490 mμ filter for carotene and a 515 mμ filter for the ferric chloride-dipyridyl reaction. Reagent blanks and 3 standards are run with each set of determinations, and the carotene correction factor is set up as in the micro method. This factor was found to be 0.39 (±.03) in our laboratory.

After subtraction of the appropriate reagent blanks from the 490 and 515 mμ densities, the calculation for tocopherol is:

$$\text{mg \% tocopherol} = (D_{515} - .39D_{490}) \times F \times \frac{3}{V}$$

where

$$F = \frac{\text{mg \% D-}\alpha\text{-tocopherol in standard}}{D_{515} \text{ of standard}}$$ (average derived from the 3 standards, 3 ml samples)

and V = Volume of plasma used.

TABLE I. Statistics—Comparison of Methods.

	Macro-method	Micro-method
Avg level (46 subjects)	1.09 mg %	1.12 mg %
Coef. of variation (std. error/mean)	3.4% (2 subjects)	4% (Quaife) (1) (3 subjects)
Mean coef. of variation between both technics (46 subjects)	$= \sqrt{\frac{\left[\frac{\text{Micro-Macro}}{\text{Micro}} \times 100\right]^2}{N(N-1)}} = 2\%$	
Recoveries (added D-α-tocopherol)	99.7%	107% (Quaife) (1)

Forty-six plasma samples were analyzed by the macro and micro methods, each in duplicate, and the results, as shown in Table I, indicate that the two methods give results which are apparently identical. The macro method is now employed in our laboratory for the analysis of blood samples from normal persons and patients with a variety of diseases, and has been found completely suitable for routine use.

Summary. Expansion of the micro method of Quaife for the estimation of blood tocopherols affords a rapid and convenient routine analytical procedure.

The authors wish to express their appreciation to the Misses Helen Petriti, Ellen Brackman, and Maeda Mayran for their competent technical assistance.

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† Presented at the meeting of the Federation of American Societies for Experimental Biology, Cleveland, O.; *Fed. Proc.*, 1951, v10, 294.

Inhibition of Gastric Secretion in Dogs by Hexamethonium. (19331)

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Hexamethylene bis-trimethylammonium chloride (hexamethonium, C6) is a ganglionic blocking agent of high potency and moderately long duration of action(1). All of its pharmacological actions have thus far been explicable on the basis of its ability to block the action of acetylcholine on sympathetic and parasympathetic ganglia(1,2). Kay and Smith(3) found that intramuscular injection of 100 mg of the iodide salt in human subjects abolished basal gastric secretion for several hours. The gastric secretory response to insulin was delayed and depressed by this dose of hexamethonium. They found that the secretory response to histamine, alcohol or meat extract was not inhibited by hexamethonium, however they measured only acid concentration in the juice and did not give values for the volume of juice secreted.

The present report presents the results of a study of the effect of hexamethonium on gastric secretion in dogs.

Methods. Four dogs with vagotomized pouches of the entire stomach and four dogs with simple gastric fistulas were used. The animals were fasted for at least 12 hours before each test and 8 experiments were made in each phase of the study. Acid secretion was stimulated by subcutaneous injection every 10 minutes of either 0.04 mg histamine diphosphate or 0.05 mg Urecholine (carbamyl β-methyl choline chloride, Merck).† The gastric juice was collected every 20 minutes, the volume was measured and the acid concentration determined by titration with 0.03 N NaOH using p-dimethylaminoazobenzene and phenolphthalein as indicators. When the rate of acid secretion had reached a stable plateau (about 2 hours after beginning injections of the stimulatory drug) hexamethonium (Bistrium, Squibb)‡ was injected intravenously in a dose of 4 mg/kg. The details of this method for studying gastric secretory inhibitors have been presented elsewhere(4).

Results. The results are presented in Table I. Hexamethonium inhibited both Urecholine and histamine-stimulated acid secretion in both vagotomized total pouch dogs and vagally innervated gastric fistula dogs. The degree of inhibition of both drugs was greater in the dogs with vagally innervated stomachs than in those with vagally denervated stomachs, although the former secreted acid at a more rapid rate. In both types of dogs the inhibition of Urecholine induced secretion was greater than for histamine. This may be related to the fact that the dose of Urecholine used evoked a lower secretory rate than the histamine.

In all instances the inhibition persisted throughout the 3hr period after injection of hexamethonium during which observations were made.

Discussion. The inhibition of secretion in the vagally innervated stomach preparations can be explained on the basis of vagal blockade, because it has been shown that vagotomy reduces the response to histamine and other stimuli(5). However, the inhibition observed in the vagally denervated preparations cannot be attributed to vagal blockade. Hexamethonium has been shown to be devoid of antihistamine and anti-acetylcholine action when tested on isolated smooth muscle(2). A theoretically possible explanation of the mechanism of inhibition by hexamethonium in the vagally denervated stomach preparation is as follows: The vagally denervated stomach is known to be capable of synthesizing acetylcholine(6). This production of acetylcholine may be, in part at least, due to the activity of post-ganglionic vagal nerves. That is, we may postulate that the post-ganglionic vagal fibers continue to form some acetylcholine after the preganglionic fibers have been severed. We may further postulate that hexamethonium would depress the formation of acetylcholine

* Jessie Horton Koessler Fellow.

† Kindly supplied by Merck & Co.

‡ Kindly supplied by E. R. Squibb and Sons.

TABLE I. The Effect of Hexamethonium (C6) on Histamine and Urecholine Stimulated Secretion in Dogs with Vagotomized Total Pouches and in Dogs with Gastric Fistulas (Vagally Innervated). Four dogs in each experiment; 2 tests on each dog.

Type of dog	Stimulant	Control secretory rate free HCl, mM/hr	% inhibition Hr after C6 1	2	3
Gastric fistula	H*	5.70 ± 1.1	47 ± 11.5	63 ± 6.3	68 ± 10.3
	U	2.70 ± .9	70 ± 7.6	97 ± 1.4	96 ± 1.8
Total pouch	H	2.50 ± .6	41 ± 1.3	31 ± 11	37 ± 5.5
	U	1.81 ± .6	44 ± 13.9	73 ± 19.9	68 ± 12.8

* H = Histamine, U = Urecholine.

by the preganglionically denervated ganglion cells. Since cholinergic stimuli potentiate the response of the parietal cell to other direct acting stimuli(7,8), a decrease in response would result. The ability of hexamethonium to suppress the spontaneous activity of preganglionically denervated ganglion cells is attested by the fact that it inhibits certain of the spontaneous movements of the isolated ileum(2).

Hexamethonium resembles atropine(9) in that both have a greater inhibitory activity in the vagally innervated than in the vagally denervated stomach. Atropine acts by blocking the effect of acetylcholine, hexamethonium, according to the present hypothesis, by depressing acetylcholine synthesis. If it is assumed that the basal rate of acetylcholine synthesis is greater in the vagally innervated than in the vagally denervated stomach then this difference in effectiveness of hexamethonium and atropine in these two types of stomach preparations would be explicable.

Summary. In dogs with gastric fistulas (vagally innervated) and in dogs with pouches of the entire stomach (vagally denervated), the secretion of acid in response to Urecholine or histamine is depressed for at least 3 hours following intravenous administration of 4 mg/kg of hexamethonium bis-trimethylammonium chloride. The inhibition is greater in the vagally innervated than in the vagally denervated stomach preparations.

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Effect of Antibacterial Substances on Fecal Clostridia Populations.* (19332)

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The reports of marked increase in the rate of gain of chicks, turkey poults and weaned pigs fed diets supplemented with antibiotics have aroused interest in the possible role of intestinal microflora on growth. In an effort to obtain information on the action of antibiotics, McGinnis and coworkers(1) have observed that the growth of *C. perfringens* is inhibited in the ceca of turkey poults fed

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diets containing 100 p.p.m. of penicillin and terramycin. Fecal samples from pigs fed diets containing 15 mg of terramycin per kg contained less than 10 *C. perfringens* per g as compared with 34 to 70 million for samples from control pigs.

The studies reported herein were conducted with weaned pigs to observe the effect of dietary antibiotics and 3-nitro-4-hydroxy phenyl arsonic acid on the population of clostridia in feces.

Procedure. Two separate experiments were conducted on the effect of dietary antibiotics and 3-nitro-4-hydroxy phenyl arsonic acid on the population of *C. perfringens* in the feces of weaned pigs. The basal diet employed for both experiments had the following composition: ground yellow corn 41%, ground oats 20%, tankage (60%) 10%, soybean oil meal 7%, alfalfa leaf meal 10%, wheat middlings 10%, and mineral 2%. In the *first experiment,* 28 weaned Chester White pigs from the Hormel Foundation herd weighing between 34 and 58 lbs were divided into 7 groups of 4 pigs each and were fed the following dietary supplements:

Group	Supplement to 100 lb of basal
1	0 g
2	1.14 g 3 nitro-4-hydroxy phenyl arsonic acid
3	4.54 g 3 nitro-4-hydroxy phenyl arsonic acid
4	.25 g procaine penicillin
5	2 g " "
6	.25 g aureomycin hydrochloride
7	2 g " "

The pigs were housed in a constant temperature farrowing barn in pens 6 x 15 ft with concrete floors and glazed tile walls. The floors were washed with water once daily, and excreta were removed at least 6 times daily. Fresh water and feed were supplied at all times. The pigs were weighed weekly for 4 weeks. All pigs were fed the basal diet during the first week and the supplemented diet during the last 3 weeks. Two fecal samples were collected from each pen 4 times a week, after the floors had been washed down with water. The fresh samples were weighed, diluted with water, and used immediately for bacterial studies.

Clostridia populations were determined by the method of Wilson and Blair(2) as modified by Thompson(3). Verification of colonies for clostridia was carried out by subculturing in iron-milk. Stormy fermentation was produced by about 80% of the colonies. In the *second experiment,* carried out over a 5-week period, 20 weaned Chester White pigs weighing between 28 and 48 lbs were divided into 5 groups of 4 pigs each. The management conditions were the same as in the first experiment. All pigs were fed the basal diet for one week during which time fecal samples were collected. Starting the second week, the pigs were fed the following dietary supplements for a period of 4 weeks:

Group	Supplement to 100 lb of basal
1	0 g
2	4.54 g 3-nitro-4-hydroxy phenyl arsonic acid
3	2 g procaine penicillin
4	2 g chloramphenicol
5	2 g aureomycin hydrochloride

Fecal samples were collected from individual pigs 5 days each week by inducing defecation by exercise or by inserting a suppository into the rectum. The bacteriological methods were the same as in the first experiment.

Results and discussion. The average daily gain and the clostridia populations of fecal samples from the first experiment are given in Table I. The pigs fed the diet containing a high level of arsonic acid (4.54 g per 100 lb of feed) gained an average of 0.93 lb per day compared to 0.78 lb for the control pigs, even though the fecal clostridia populations were essentially the same for the two groups. The clostridia populations of the feces of the group fed the low level of arsonic acid (group 7) were also in the same range and the growth rate was the same as that of the control pigs. The animals fed diets containing penicillin (groups 4 and 5) and 2.0 g aureomycin per 100 lb of feed (group 2) had the lowest populations of fecal clostridia during the last 2 weeks, but they did not gain any faster than the pigs fed the high level of arsonic acid.

The results of the second experiment are given in Table II. All the supplemented groups made better weight gains than the control group.

TABLE I. Effect of Dietary Antibiotics and 3-Nitro-4-Hydroxy Phenyl Arsonic Acid on Population of *C. perfringens* in Feces of Pigs.

Group No.	Supplement per 100 lb basal diet (g)	Avg daily gain,* lb	Avg No. of *C. perfringens* per g fresh feces ($\times 10^3$) 1st wk	2nd wk	3rd wk	4th wk
1	0	.78	487	90	19	17
2	2 aureomycin hydrochloride	.87	53	30	1.9	4.3
3	.25	.81	373	13.5	7.6	13.6
4	2 procaine penicillin	.90	20	1.4	.4	.1
5	.25	.89	3046	18	.3	2.3
6	4.54 3-nitro-4-hydroxy phenyl arsonic acid	.93	500	31	69	12
7	1.14	.77	40	17.5	22	17

* During the last 3-week period when supplements were fed.

TABLE II. Effect of Dietary Antibiotics and 3-Nitro-4-Hydroxy Phenyl Arsonic Acid on Population of *C. perfringens* in Feces of Pigs.

Group No.	Supplement per 100 lb basal diet (g)	Avg daily gain,* lb	Avg No. of *C. perfringens*/g fresh feces ($\times 10^3$) 1st wk	2nd wk	3rd wk	4th wk	5th wk
1	0	1.05	2960	614	29.4	61	126.4
2	2 aureomycin hydrochloride	1.19	3975	798	8.9	15	3.5
3	2 procaine penicillin	1.38	5900	18900	325.9	31.9	3.8
4	2 chloramphenicol	1.23	134	6051	15	25.8	21.5
5	4.54 3-nitro-4-hydroxy phenyl arsonic acid	1.31	3280	639	22.1	20	30

* During last four wk of experiment when supplements were fed.

In general the population of clostridia in the feces decreased for all groups during the test period and there was no apparent correlation between the rate of gain and population of the fecal samples.

There was considerable variation in the population of the fecal samples collected from the same lot of pigs on different days. Also, there was day-to-day variation in the samples from the same animal. The average daily gains for the pigs in each group varied considerably, in some cases. Because the number of pigs per group was small, the differences in rates of gain of pigs in the supplemented lots over those of control pigs were not significant except for group 6 of Table I. In most other cases, the differences approached significance at the 5% level.

Summary. The results in 2 separate experiments suggest that populations of fecal clostridia can be altered by feeding antibiotics, but growth of the host animal is not directly related to the population of fecal clostridia.

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Toxicity of Dimethyl Selenide in the Rat and Mouse. (19333)

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(Introduced by M. Mason Guest.)

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The toxicity of inorganic selenium varies considerably according to its ionic state. According to Franke and Potter(1), the order of toxicity of the inorganic seleniums from the highest to the lowest is selenate, selenite, and selenide. Elemental selenium is almost nontoxic. The *minimum fatal dose** of sodium selenate injected intraperitoneally in the rat was found to be 5.25 to 5.75 mg Se per kg of body weight(2). Similarly, the toxicity of organo-selenium compounds is variable(3-6). A study of various seleniferous diets, as reported by Franke and Painter(7), showed the toxicity of selenium from different sources to be in the following order: wheat, >corn, >barley, >selenate, >selenite, >selenide, and >elemental selenium. They observed that there was little difference in the toxicity of selenium as it occurs in the different grains, but the naturally-occurring selenium was found to be definitely more toxic than inorganic selenium. Since naturally-occurring selenium has been shown to be in the protein fraction of seleniferous grains(8), and it has been postulated that selenium could replace sulfur in methionine and cystine(9), the toxicity of the selenium analogs of these amino acids is of particular interest. The *minimum fatal dose** of selenium in the form of selenium-cystine when injected intraperitoneally into the rat was found to be 4.0 mg Se per kg of body weight(4).

It was noted in previous experiments(10) in which studies were carried out to investigate the time-excretion of injected dimethyl selenide, that relatively large amounts of dimethyl selenide (600 mg Se per kg of body weight) could be administered to rats without any apparent deleterious effects. A systematic search of the literature revealed that no toxicity studies of dimethyl selenide had been reported. Therefore, it was the purpose of the experiments presented here to determine the *median lethal dose* (L.D. 50) of dimethyl selenide in the rat and mouse.

Experimental. Dimethyl selenide was prepared according to the method of Bird and Challenger(11). Elementary carbon and hydrogen analyses† revealed the following results:

$(CH_3)_2Se$ (109.03)	Carbon, %	Hydrogen, %
Theoretical	22	5.55
Found	23.3	5.69

The mercuric derivative $(CH_3)_2Se \cdot HgCl_2$ (12) melted at 151-153°C. The $(CH_3)_2Se$ had a boiling point at 53°C and had a specific gravity of 1.41 at 15°/4°C. The preparation was injected intraperitoneally into the animals, using a tuberculin syringe for the rats and a syringe specially constructed from a narrow bore serological pipette for the mice. Sixty-three mice of mixed sex with an average weight of 25 g, and 34 male rats with an average weight of 311 g were administered doses of dimethyl selenide which are recorded in Table I. The mortality was determined at

TABLE I. Mortality of Mice and Rats Injected with Dimethyl Selenide.

Dose $(CH_3)_2Se$, g/kg	No. of animals	Mortality—24 hr, %	Mortality—48 hr, %
1.4	15 m*	26.7	33.3
1.4	5 r	0	0
2.1	15 m	66.7	100
2.1	11 r	45.5	54.5
2.8	15 m	86.7	93.3
2.8	5 r	80	80
3.5	14 m	92.9	92.9
3.5	13 r	84.6	84.6
7	4 m	100	100

1 ml $(CH_3)_2Se$ = 1.018 g Se as $(CH_3)_2Se$, or 1.408 g $(CH_3)_2Se$ at 15°/4° C.
* m = mice, r = rats.

* The minimum fatal doses were taken as the smallest doses which would kill 75% or more of the animals in less than 2 days.

† Analyses by the Clarke Microanalytical Laboratory, Urbana, Ill.

24 and 48 hours after the time of injection.

Results and discussion. Within one to 2 minutes following injection of dimethyl selenide near the median lethal dose, the animals entered a state of hyperpnea with the elimination of an overwhelming garlic-like odor on the breath. Convulsions in the mice, but not in the rats, were common. This was usually followed by exitus within a few hours, with the fatal doses, although some animals died as late as 36 hours after injection. In all cases, the signs of hyperpnea were gone after the first 2 or 3 hours.

Median lethal doses (L.D. 50) for the rat and mouse at 24 hours were determined (13,14) by plotting per cent mortality on probit against log dosage graph paper. It was found that the L.D. 50 for 24 hours for the mice was 1.3 g Se as dimethyl selenide (1.8 g dimethyl selenide) per kg of body weight. That for the rats was 1.6 g of Se as dimethyl selenide (2.2 g dimethyl selenide) per kg of body weight. It will be noted that these figures are several times greater than the minimum fatal doses for either sodium selenate (5.25 to 5.75 mg Se per kg)(2), or selenium-cystine (4 mg Se per kg of body weight)(4).

In relation to the selenium detoxification mechanism, it is of particular interest to point out that after the administration of sodium selenate, which is a relatively toxic compound, selenium rapidly appears in the respiratory gases(15) in the form of dimethyl selenide (10), which is a sparingly toxic compound. Thus, it would appear that the animal organism when treated with a toxic form of selenium, converts it in part to a less toxic compound which is readily excreted via the lungs.

Summary. The median lethal dose (L.D. 50) of dimethyl selenide was determined for the mouse and rat. It was found, after intraperitoneal injection of dimethyl selenide, that the median lethal dose for the mouse was 1.3 g of Se as dimethyl selenide (1.8 g dimethyl selenide) per kg of body weight, and 1.6 g Se (2.2 g dimethyl selenide) per kg of body weight for the rat.

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Leucocytosis in Vitamin E Deficient Rabbits.* (19334)

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Previous reports from this laboratory have indicated an effect of vit. E deficiency on nucleic acid metabolism(1,2). It was also noted that E-deficient monkeys exhibited a leucocytosis which responded to therapy with alpha tocopherol(1). This paper reports the results of a study of the effect of vit. E deficiency on the peripheral leucocytes of rabbits. The data to be presented show that E-deficient rabbits exhibit a leucocytosis which

TABLE I. Peripheral Leucocytes in Control and Vitamin E Deficient Rabbits.

Diet	No. of rabbits	Peripheral leucocytes, thousands/μl. mean ± stand. error		
		Total WBC	Lymphocytes	Granulocytes
Chow	10	10 ± 1.1	5.8 ± .7	3.5 ± .6
Ferric chloride treated — E	4	25.9 ± 6.9	4 ± .8	19.5 ± 7
" " " + E	3	5.8 ± .5	2.8 ± .3	2.6 ± .2
Purified — E	7	22.4 ± 1	3.7 ± .7	17.1 ± 1.1
" + E	5	10 ± 1.3	5.3 ± .7	3.8 ± 1.3

is the result of a greatly increased number of circulating granulocytes.

Materials and methods. White New Zealand rabbits of both sexes and weighing approximately one kilogram were placed on the experimental diets. In one series the rabbits were given the ferric chloride treated diet described by Mackenzie and McCollum(3). In another series the animals were fed the purified diet previously described(2). Controls for both series were given the same diet plus supplements of 2 mg of alpha tocopherol acetate daily per kilo body weight. The supplement was administered orally from a corn oil solution by means of a syringe with a blunt needle. Additional controls were given a diet of commercial rabbit chow. *Blood counts* were taken after the rabbits receiving the deficient diets were unable to right themselves when placed on their sides. Controls were bled at the same time. Rabbits exhibited this symptom of muscular dystrophy after 2 to 4 weeks on the deficient diets. Blood counts were made by standard hematological procedures on blood taken from the marginal ear vein. There were no differences in red cell counts, hemoglobin, or hematocrit values and the data are not presented.

Results and discussion. The data in Table I show the circulating granulocytes are greatly elevated in vit. E deficient rabbits when compared either to their respective controls or to rabbits receiving the chow diet. In recovery experiments it has been found that when the deficient rabbits are recovered with alpha tocopherol the peripheral blood picture returns to normal.

These experiments do not prove that the leucocytosis which is exhibited by rabbits suffering from nutritional muscular dystrophy is a direct result of vit. E deficiency. The peripheral leucocytosis may merely reflect a leucocytic infiltration of the dystrophic muscle and not be specific for E deficiency. The elevated peripheral white cell count is of some interest however since it has been shown that the deficient rabbits synthesize extra amounts of creatine(4) and data from this laboratory have suggested a relationship between creatine and white blood cell production(5,6).

Summary. The peripheral granulocytes are greatly elevated in vit. E deficient rabbits.

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Immunophysiology. Increase in Radiosodium Space of Adrenal Glands in Rabbits Sensitized with Human Plasma.* (19335)

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Since von Pirquet and Schick published their description of serum sickness(1), many studies concerning the relationship of certain histologic findings to immunologic phenomena have been reported(2-5). Such anatomic changes have been well delineated, but the physiologic alterations which are induced by antigen-antibody reactions are still poorly understood. It has been found previously that in rabbits sensitized with human plasma shifts in the distribution of body fluids occur at the time humoral antibody is first detected. The data were interpreted as indicating that a severe immune reaction may be associated with changes in the permeability of membranes (6). The methods employed in these studies did not permit localization of the observed changes to any specific tissues or organs.

The present experiment was undertaken in order to determine the site of the maximum alteration in fluid distribution following the injection of a large dose of human plasma into rabbits. Radioactive sodium (Na^{24}) was used as a tracer.

Materials and methods. Domestic rabbits of mixed breeds, weighing from 2 to 4 kg each, were placed in individual cages and fed a stock diet. Water was given without restriction. The same pool of human plasma was used as the sensitizing antigen and as antigen for the precipitin test. Another pool of human plasma was denatured by being heated to 100°C for 10 minutes. The filtrate of heat-denatured plasma was diluted with physiologic saline solution to the original volume. The methods for the determination of the plasma volume, thiocyanate space, radiosodium space, tissue radiosodium content, and tissue water content have been described previously(7). The appearance of humoral antibody was detected by a qualitative ring precipitin test. The human plasma, diluted 1:100 in physiologic saline solution, was used as antigen to overlay an equal volume of undiluted rabbit serum in 2 x 20 mm test tubes.

Plan of the experiment. Eighteen rabbits were divided into 3 groups of 6 each. *Group 1* (test) and *Group 2* (control) consisted of 6 rabbits each. The animals in the 2 groups were paired as to sex and body weight. On each rabbit, two baseline determinations of the plasma volume, the hematocrit, and the thiocyanate and radiosodium spaces were made 6 to 7 days apart. Each of the animals in Group 1 then received intravenously 10 ml of human plasma per kg of body weight, 5 ml/kg being administered on each of 2 successive days. The animals in Group 2 did not receive injections of human plasma, but were otherwise treated identically. Beginning on the fourth day after the injection of the initial dose of antigen, precipitin tests were performed daily on all rabbits in both groups. On the day when humoral antibody first appeared in an animal in Group 1, the volume determinations were repeated on the animal in Group 2 which had been paired with it, and the 2 rabbits were then sacrificed by exsanguination. For each animal the tissue radiosodium space was determined on 2 portions (roughly 1-2 g each) of the following: cartilage, kidney, skin, tendon, lung, subcutaneous connective tissue, heart, appendix, stomach, adrenal, liver, spleen, abdominal muscle, diaphragm, and gastrocnemius muscle. The total water content was determined on one portion of each tissue. *Group 3,* which served as an additional control, consisted of 6 rabbits whose sex and weight distribution was comparable to that of the rabbits in the preceding two groups. Each animal received by intra-

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venous injection 10 ml of denatured human plasma per kg of body weight, 5 ml/kg being administered on each of 2 successive days. No volume determinations were made in this group, since no changes had been detected in Groups 1 and 2. Six and a half days after completion of the injection of denatured plasma—the average interval at which precipitins were first detected in Group 1—the animals were sacrificed by exsanguination for tissue analyses. At that time the precipitin tests were negative to both denatured and undenatured plasma. The following organs were studied: adrenal, kidney, stomach, and skeletal muscle.

Statistical analysis. The total blood volume, plasma volume, thiocyanate and radiosodium spaces were recorded in milliliters per kg of body weight. For each animal the difference between the mean of the two baseline determinations and the single value obtained on the day the precipitin test first became positive was calculated. The mean difference between the baseline and response values was then obtained, and the significance of the mean difference was tested by the following formulae in which $\bar{d}$ = mean difference, $s\bar{d}$ = standard error of the mean difference, S = summation of, n = number of animals:

$$t = \frac{\bar{d}}{s\bar{d}} \qquad s\bar{d} = \sqrt{\frac{Sd^2 - \frac{(Sd)^2}{n}}{n\,(n-1)}}$$

The calculated value for "t" was compared with the value at (n—1) degrees of freedom, as obtained from standard tables. "t" was considered significant if $P < 0.01$. The results of the tissue analyses were analyzed by groups. The mean values for tissue water content and radiosodium content were obtained for each group and their respective standard errors were then calculated. The significance of the difference between the means was tested by the following formulae:

$$\bar{d} = \bar{x}_1 - \bar{x}_2 \qquad s\bar{d} = \sqrt{s\bar{x}_1^2 + s\bar{x}_2^2}$$

$\bar{x}$ = group mean
$s\bar{x}$ = stand. error of mean

Results. Precipitins to undenatured human plasma developed in the animals in Group 1

TABLE I. Fluid Spaces in Rabbits Given Injections of Human Plasma and in Paired Uninjected Control Animals.

Group			Wt (kg)	Radiosodium space (ml/kg)	Thiocyanate space (ml/kg)	Plasma vol. (ml/kg)	Hematocrit (vol. %)	Total blood vol. (ml/kg)
1	Test animals inj. with human plasma, 10 ml/kg	Baseline	3.07 ± .60	256 ± 15.7	216.5 ± 19.6	41 ± 4.2	40.8 ± 2.4	69.8 ± 8.5
		Response*	3.39 ± .45	261 ± 14.2	221.3 ± 28.8	43.7 ± 8.9	37.8 ± 2.8	70.5 ± 16
2	Controls not inj. with plasma	Baseline	3.57 ± .12	249 ± 11	209.2 ± 10.7	39.8 ± 6.7	40.3 ± 4.1	66.5 ± 9.5
		Response†	3.66 ± .47	249 ± 10.9	207.2 ± 18.9	37 ± 4.05	39.5 ± 3.3	61.5 ± 7.8

All values are expressed as mean ± stand. dev.
* Values obtained on day when humoral antibody first appeared (mean of 6.5 days after initial inj. of human plasma).
† Values obtained at a time interval corresponding with appearance of humoral antibody in Group 1.

(test) at a mean of 6.5 days after the initial injection of antigen. No precipitins to undenatured or denatured plasma developed in the animals in Groups 2 or 3. When the average of the mean baseline values at the time of sacrifice were compared with the average of the mean baseline values, no significant difference was found in the weight, plasma volume, hematocrit, total blood volume, thiocyanate space, or total radiosodium space in any group (Table I).

Comparison of the results of the tissue analyses on the animals in Groups 1 and 2 shows the radiosodium space in the adrenal glands of the sensitized animals to be significantly increased, without an accompanying increase in total water content (Table II). No significant difference in the radiosodium space or the water content of any of the other tissues or organs was found when Group 1 is compared with Group 2.

The results of the tissue analyses in Groups 1 and 3 likewise reveal the radiosodium space to be significantly increased only in the adrenal glands of the animals injected with undenatured plasma as compared with those given denatured plasma. No significant difference in the total water content of the gland was found. The kidney, stomach, and skeletal muscle revealed no change in radiosodium space or total water content.

Comment. The development of humoral antibody in all animals injected with undenatured plasma (Group 1) indicates that a certain degree of sensitization was induced. Since no generalized physiologic alterations developed it must be assumed that this sensitization was minimal. The fact that changes in the tissue radiosodium space were limited to the adrenal glands of those animals injected with untreated human plasma and were not present in those given denatured plasma or no antigen at all suggests that this gland may play some vital role in the maintenance of homeostasis. It may be suggested that the change in the adrenal gland may be a compensatory response to the harmful effects of an antigen-antibody reaction. From the data available, it is not possible to determine whether this increase in the radiosodium space of the adrenal gland was due to interstitial edema, to an increase in the isotope content within the parenchymal cells, to a relative decrease in parenchymal tissue, or to a combination of these factors.

TABLE II. Radiosodium Space and Total Water Content of the Adrenal Gland in Sensitized and Control Rabbits. Six animals in each series.

Group		Mean values: Radiosodium space (% of wet wt)	Mean values: Water content (% of wet wt)
1	Inj. with undenatured plasma	19.06	63.03
2	Uninj. controls	14.98	65.75
3	Inj. with denatured plasma	11.26	56.80

Summary of statistical analyses

Groups	Radiosodium space $\bar{d}$	Radiosodium space $S\bar{d}$	Radiosodium space t	Water content $\bar{d}$	Water content $S\bar{d}$	Water content t
1 vs. 2	4.08	.63	6.48*	2.72	4.74	.57
1 vs. 3	7.80	1.32	5.91*	3.72	1.20	3.10
2 vs. 3	3.72	1.20	3.10	8.95	3.94	2.27

* Statistically significant. $P = <.01$.

Summary. Rabbits sensitized with human plasma (10 ml/kg) developed humoral antibody following a mean interval of 6.5 days. No changes were noted in the blood volume or in the thiocyanate or radiosodium space at that time. Tissue analyses revealed the radiosodium space to be significantly increased in the adrenal gland of those animals given injections of untreated plasma, as compared with animals given denatured plasma or no antigen at all. No such increase was noted in any of the 14 other tissues or organs studied. No significant differences in the water content of any of the tissues or organs were found.

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Absorption and Toxicity of Dieldrin. (19336)

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Dieldrin,* a relatively new insecticide, is very satisfactory for many purposes. It is compatible with most agricultural fertilizers, herbicides, fungicides, and insecticides. It shows promise for the control of many insects, particularly household pests, animal parasites, cotton insects, and soil inhabiting larvae. Its usefulness is enhanced by its high toxicity to insects, stability in acids and bases, and failure to impart flavors when used on fruits and vegetables.

Dieldrin is known to be toxic to warm blooded animals; hence it was deemed desirable to determine (a) its toxicity when applied at various concentrations, (b) its chronic toxicity when fed orally, (c) the amount of insecticide accumulated in the organs of the laboratory animals, and (d) whether sprayed cows eliminated dieldrin in their milk.

Experimental animals. The rats and rabbits used as experimental animals in determining the toxicity of Dieldrin were kept in wire cages and observed for abnormal actions and other symptoms of toxicity. Jersey cows under normal farm conditions were used to determine the amount of Dieldrin excreted in the milk. At death or at the termination of an experiment, an autopsy was performed on all small animals. Liver and kidney samples from the individual rats of each group were pooled, placed in sealed jars, quickly frozen, and stored until analyzed. The milk samples were treated immediately with formaldehyde and refrigerated. The Dieldrin was determined as organic chlorides according to the method adapted by Carter(1), the liberated chlorine being determined by the McLean and Van Slyke(2) method.

Toxicity of external applications of Dieldrin. Rabbits were dipped in a dilute emulsion of xylene, Triton x-155, Dieldrin, and water. The weight of the animal, mg/kilo Dieldrin used, survival period, and p.p.m. Dieldrin in the liver and kidney are given in Table I. These data show that all animals absorbed Dieldrin and retained it in their livers and kidneys. Refusal to eat and drink and listlessness were constant symptoms. When lethal doses were given, 14 to 100 p.p.m. Dieldrin was found in the livers and 21 to 165 p.p.m. in the kidneys. Mature rabbits receiving 70 mg/kilo or more at weekly intervals died; 50 mg/kilo or more caused death in immature rabbits. Symptoms noted were salivation, grinding of teeth, and spasms. Animals receiving less than 30 mg/kilo showed no symptoms during the 70 days of the experiment. Lower concentrations produced no outward symptoms. Three lots of immature rats were dipped respectively in .125%, .187%, and .250% Dieldrin emulsions once a week. Resulting data are listed in Table II. The average weight of animals increased gradually. None of the animals dipped in the .125% emulsion died. One rat dipped in the .187% emulsion died after 3 treatments, and one in the .250% after 2 treatments; indicating that individual animals have different tolerances. The Dieldrin found in the liver ranged from 25 to 35 p.p.m. and in the kidney from 42 to 52 p.p.m. The concentrations of insecticide applied to these experimental animals were many times the amount normally recommended for practical insect control.

Oral toxicity of Dieldrin. The oral toxicity to rabbits was determined by force feeding Dieldrin in capsules once a week for 25 weeks or as long as the animal survived. Results are given in Table III. Rabbits numbered 3, 4, and 18, receiving the highest dosages, had convulsions and ground their teeth excessively. Opisthotonos was followed by calmness, impaired vision, and bleeding of the

* Dieldrin is the name for the insecticidal product containing not less than 85% of 1, 2, 3, 4, 10, 10-hexachloro-6, 7-epoxy-1, 4, 4a, 5, 6, 7, 8, 8a-octahydro-1, 4, 5, 8-dimethanonaphthalene.

TABLE I. Nine Rabbit Dipping Experiments. (Each animal was treated once a week).

Wt (g)	mg Dieldrin per kilo body wt	Application period (wk)	Days of survival	P. P. M. Dieldrin Liver	P. P. M. Dieldrin Kidney
3675	250	1	4	82	165
3500	250	1	6	48	21
3550	250	1	8	—	111
3575	250	1	2	14	38
3450	250	2	11	73	57
3630	200	2	9	92	148
2235	80	3	15	100	135
1475	70	2	10	68	162
3560	30	10	70*	12	12

* Animal was sacrificed.

TABLE II. Rat Dipping Experiments. Average Weight in g. (Dipped weekly).

Sol.	Weeks 1	2	3	4	5	6	7	8	9	10	P.P.M. Dieldrin* Liver	Kidney
.125%	285	276	279	309	325	321	374	379	392	†	25	42
.187%	290	268	262	310	345	355	370	†			30	51
.250%	262	277	325	316	340	350	353	†			35	52

* Pooled samples. † Rats sacrificed.

TABLE III. Eight Rabbit Feeding Experiments.

Beginning wt (g)	Final wt (g)	Sex	mg Dieldrin fed per wk	Feeding period (wk)	P.P.M. Dieldrin Liver	Kidney
3150	3400	♀	0	15	0	0
3575	4600	♀	30	25	13	6
3500	3170	♀	60	13	49	77
3575	2825	♀	110	11	76	181
975	3363	♀	0	14	0	0
950	3275	♀	10-20*	14	—	-
1015	3300	♂	20-40*	14	23	48
875	3075	♂	35-70*	13	88	201

* Amount of Dieldrin fed doubled from 9 to 14th week.

gums. Weight gains were not appreciably affected until the rabbits received 60 mg/kilo or more. Dieldrin found in the liver ranged from 13 to 88 p.p.m. and in kidneys from 6 to 201 p.p.m. Rabbit No. 2, which received 30 mg per week for 25 weeks, did not die. Rats were weighed and force fed weighed quantities of Dieldrin once a week as long as they survived, or until the termination of the experiment. Lot I served as a control. Lot 2, receiving 15 mg Dieldrin per animal per week, made gains comparable to the controls. Lot 3 received 30 mg per rat per week, equivalent to 150 mg/kilo body weight. One rat died the second week, one the fourth, one the fifth, and the last one the eleventh week. The average growth rate was comparable until the time of death.

Excretion of Dieldrin in milk of dairy cattle. In determining the amount of Dieldrin eliminated in the milk of cows sprayed for parasite control, a herd of dairy animals under normal farm conditions was used. Each animal was sprayed with one gallon of 0.1% spray mixture at approximately 3 week intervals for tick control. Pooled milk samples were taken during milking. Following spraying, the concentration of Dieldrin in the milk gradually increased to a maximum of 5.9 p.p.m. during the first 3 weeks and then decreased steadily despite continued applications of Dieldrin.

Observations. Toxic symptoms in rabbits, such as excessive salivation, grinding of the teeth, rolling of the eyes, muscular twitching, and convulsions were observed. Later the animal became listless, did not drink, and appeared to be blind. Except for the death of 2 of the rats, which were dipped, no abnormalities were noticed. Sprayed cattle showed no symptoms.

Mature rabbits given 60 mg/kilo body weight or more at weekly intervals died; an immature rabbit died after repeated weekly treatments of approximately 50 mg/kilo body weight. Younger animals generally were more susceptible to insecticide poisoning than older ones. Rats given approximately 75 mg/kilo body weight were not affected after repeated weekly treatments. Those given 150 mg/kilo body weight died after repeated weekly treatments. This would seem to indicate that the lowest toxicity for rats falls between these 2 concentrations, considerably above the amount causing death in rabbits. The accumulation of Dieldrin in the organs of the animals treated indicates that repeated treatments may be potentially dangerous.

Conclusions. 1. Dieldrin produced chronic toxicity in rabbits when it was applied once a week at 70 mg/kilo body weight. Less than 30 mg/kilo produced no symptoms. 2. Only 2 of 11 rats dipped in .125, .187, and .250% Dieldrin spray solutions died. 3. Dieldrin produced chronic toxicity in mature rabbits when fed orally approximately 60 mg/kilo body weight. Similar feeding of approximately 150 mg/kilo body weight was fatal to rats. 4. Liver and kidneys of all animals treated and analyzed were found to contain Dieldrin. The amount recovered in these organs was generally in proportion to the amount fed per week, not the duration of feeding. 5. Analyses of milk from cows sprayed with Dieldrin for external parasite control indicated a maximum of 5.9 p.p.m., which was gradually decreased regardless of continued application.

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Calcification VI. Adenosinetriphosphate Content of Preosseous Cartilage.* (19337)

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Enzymes acting on organic phosphate(1,2), particularly those involved in phosphorylative glycogenolysis(2,3), have received serious attention among the "local factors" proposed as being responsible for initiating the process of mineralization. While the existence of glycogen(2-4) and presumably some of the enzymes in the cycle has been indicated(2) the presence of adenosinetriphosphate (ATP), required in such a cycle has not been demonstrated. Cartier(5,6), however, has indicated that if ATP were present it might considerably enhance the degree of mineralization. He reported that added ATP greatly enhances *in vitro* calcification of embryonic sheep bone cartilage.

The purpose of the present investigations was to establish the existence of ATP in bone cartilage. As will be shown in this paper, ATP does exist in significant amounts in the bone cartilage of the rat; furthermore, there appears to be a correlation between the preservation of the calcifying mechanism by calcium and the ATP level.

Procedure. Lutwak-Mann(7) was not able to detect either acid hydrolyzable phosphorus nor significant quantities of other forms of organic phosphorus in trichloracetic acid extracts of articular cartilage. This must be related to the fact that the inorganic phosphorus content of cartilage is very high as compared to the organic phosphorus. It is apparent that if organic phosphorus is to be detected, methods other than those heretofore employed must be used. We decided to use the enzymatic procedure developed by one of

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TABLE I.

bone sections placed in 3 cc cold 10% TCA, let stand 30 min in cold. Centrifuge

add 3 cc 5% TCA, stand 15 min. Centrifuge

dissolved in 2 cc 30% KOH heat in boiling water bath, cool. Add 1 vol 95% alcohol, stand overnight on ice. Centrifuge

combine, neutralize, add ½ cc 25% $BaAC_2$ and 1 vol 95% alcohol, stand overnight on ice. Centrifuge

glycogen wash with 95% alcohol. Centrifuge — discard

dissolve in 3 cc water and 2-3 drops 2 N HCl put through amberlite column (activated with Na) containing 2½ g of amberlite. Check for complete removal of barium, neutralize, make to volume for Deaminase determination (fraction A). — discard

glycogen, hydrolyze in 2 cc 1 N HCl for 1 hr in boiling water bath. Neutralize to phenolphthalein with NaOH make to vol. Do Nelson glucose determination — discard

us for the detection of ATP(8). In the first experiments carried out to detect ATP, approximately 6-week-old animals, both on normal and rachitic diets, were anesthetized with nembutal (5 mg/100 g of body weight), the tibiae exposed, and sections of the heads removed. Tissue from 2-6 animals was homogenized in 3 ml of cold 10% trichloracetic acid (TCA), and filtrates prepared in accordance with the scheme shown in Table I. In later experiments, as indicated in Table I, the sections were soaked in cold TCA for longer periods of time without homogenization. Results obtained with the soaking technics were not significantly different from those after homogenization with respect to ATP, but gave much lower levels of inorganic phosphorus. In the experiments on calcification which are reported here, tissue sections from each animal were split among two groups and placed on glass hooks (2-3 sections on a hook) in Erlenmeyer flasks to which the incubating solutions were added(9). The hooks were made from capillary tubing, the ends of which were inserted into holes bored in paraffined cork stoppers. This was done so that individual variations among different animals could be avoided and insured both sides of the sections coming into contact with the solution instead of only one as in the case of sections placed at the bottom of the flasks. Experiments were also carried out on the glycogen content of the slices after different periods of incubation. Glycogen was precipitated according to the method described in Umbreit, Burris, and Stauffer(10). Glucose was measured by the method of Nelson(11). In a few runs, glycogen was estimated by the procedure of van Wagtendonk(12).

Results and discussions. Ultraviolet spectra

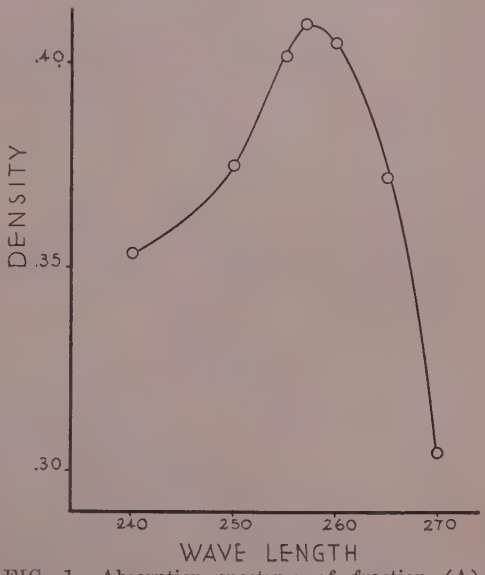

FIG. 1. Absorption spectrum of fraction (A) (see Table I) prepared from epiphyseal cartilage.

TABLE II. ATP Content of Cartilage from Normal and Rachitic Rats. (ATP expressed as adenine. The value per rat represents both tibiae.)

Exp. No.	ATP	No. rats	γ/rat	Condition*
1	34.5	2	17.2	R
2	22.5	2	11.2	R
3	28.5	3	9.2	N
4	58	5	11.6	R
5	59	6	9.8	R
6	47.4	4	11.8	N
7	66.6	6	11.1	R

* R = rachitic; N = normal.

obtained from filtrates prepared as described in Table I (fraction A) have absorption maxima characteristic of the adenine nucleotides with a slight shift towards the lower wave lengths (Fig. 1). Enzyme determinations in addition showed that ATP was present. AMP and ADP were virtually absent. The results of the first group of runs on both normal and rachitic animals are shown in Table II. It is clear from these results that cartilage contains clearly measurable quantities of ATP. (Comparative data on the relative content of cartilage as compared to other tissues will be presented below.)

The next group of studies was concerned with changes in ATP levels in the basal solution used in studies of calcification *in vitro* (13). This solution is free of calcium and phosphate. Two groups of experiments were carried out. one in which the sections were incubated in the basal solution and the other in which the basal solution was supplemented with calcium chloride (2.5 millimoles per liter). Data from a typical experiment are shown in Table III. It is clear that in the early intervals the ATP levels are depleted more rapidly from sections placed in basal solution than from sections placed in basal solution containing Ca^{++} ions. These results are interesting in the light of the observations that calcium ions in the basal solution exert a protective action against deterioration of the calcifying mechanism (9,14). These data suggest that there may be a relationship between the ATP levels and the ability to calcify.

In other studies carried out by Marks and Shorr (3), a correlation was established between glycogen content (as measured by iodine staining) and subsequent ability of cartilage sections to calcify. We measured glycogen levels in the TCA residues of the slices on which we carried out the ATP analyses. Approximately a 50% reduction in the glycogen content of both sets of sections occurred by the end of 12 hours. In contrast to the results obtained with ATP, however, we could find no significant differences between the glycogen content of slices incubated in basal solutions or solution supplied with Ca^{++} (Table III). Since our analytical procedure depends upon the conversion of glycogen to glucose, it was possible that what we were measuring was reducing sugar derived from some other source, for example, from chondroitin sulfate, a common constituent of cartilage. We ruled out this possibility in the following way: chemical determination of glycogen directly using the procedure of van Wagtendonk (12) gave results of the same order of magnitude as that obtained after hydrolysis. If anything, the results were higher. Chondroitin sulfate† in addition did not precipitate with glycogen under our experimental conditions. These conclusions do not imply that glycogen is not involved in calcification, but indicate that under the conditions of our experiment, ATP, rather than glycogen, was probably limiting. Should further investigation indicate that the ATP is in the matrix, one of the difficulties with the theory of Robison (15) as modified by Robison and Rosenheim (1) would be eliminated. This theory visualizes the deposition of bone salt in the following manner:

$$\text{Organic } PO_4 \xrightarrow[\text{phosphatase}]{} R + PO_4 \xrightarrow[\text{Ca ion}]{\text{2nd mechanism}} \text{Bone salt}$$

The major difficulty with the theory was that hitherto no appreciable amounts of organic phosphate were found in the matrix. This relative lack of organic phosphate is what led Gutman and his coworkers (2,16,17) to investigate glycogen and phosphorylative glycogenolysis in relation to the calcifying mechanism as a source of readily renewable organic phosphate.

† Obtained through the courtesy of Dr. Saul Roseman, Department of Pediatrics and Biochemistry, University of Chicago.

TABLE III. ATP and Glycogen Content of Slices Incubated on Glass Hooks in Basal and Calcium Chloride (2.5 mMols per liter) Supplemented Solutions for Varying Periods of Time.

	Basal Ca			Basal sol.			mg % glycogen	
Time, hr	mg % ad as AA	mg % ad as ADP	mg % ad as ATP	mg % ad as AA	mg % ad as ADP	mg % ad as ATP	Basal sol., Ca	Basal sol.
0	.0	1.1	8.3	.0	1.1	8.3	349	349
3	.3	.9	5.4	.0	1.8	1.6	291	226
6	.0	.9	3.8	.7	.8	.9	219	206
12	.7	.4	2.2	.7	.4		185	188

One last point of interest deserves mention. Expressed as adenine, the ATP level of cartilage is approximately 9 mg %. Expressed as ATP, this would correspond to about 35 mg %. ATP values obtained for liver, kidney, brain and muscle in our laboratory were respectively 40, 29, 50 and 300 mg %. Cartilage therefore has an ATP level in the same range as kidney, liver, and brain.

Summary. ATP has been identified in TCA filtrates of epiphyseal cartilage of rat tibiae. The levels present are approximately those found in kidney, liver, and brain. The disappearance of ATP from cartilage slices incubated in basal solution is retarded initially by small amount of calcium ion. This may account in part for the prevention by calcium of the deterioration of the calcifying mechanism as reported earlier(14). A similar correlation does not occur in the case of glycogen.

We wish to acknowledge the valuable assistance and suggestions of Dr. Harry Goldenberg and Mr. Albert Hanok, and Miss Mona Oser and Dr. Benjamin Oser of the Food Research Laboratories, Inc., for providing many of the experimental animals used in these studies.

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Effect of Terramycin and Vitamin B_{12} on Hatchability.* (19338)

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(Introduced by E. R. Norris.)

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Terramycin has been shown to support growth of swine and poultry when added to a complete diet including vit. B_{12}(1,2). When fed to lactating females, it also increased the weaning weights of rats as shown by the work of Stern and McGinnis(3). Elam *et al.*(4) using cross-bred chickens, concluded that penicillin improved egg production. Their results showed that the injection of 1 μg vitamin B_{12} per bird per week gave 38% egg production as compared with 50% for the combination of vit. B_{12} and penicillin (33 mg/kg). These rates of production are not very outstanding. Hatchability of fertile eggs for the 2 treatments was 72 and 80%, respectively. The vit. B_{12} content of the liver, kidney, and egg yolk for the 2 treatments in their study was not appreciably different, but hatchability and, to some extent, egg production were improved by the penicillin supplement.

The purpose of the present study was to determine the effect of terramycin on egg production and hatchability when fed alone and in combination with vit. B_{12}.

Procedure. White Leghorn pullets, 8 weeks of age, were divided into 4 groups of approximately 90 birds each. The pullets were kept on range and fed mash and whole wheat free-choice during the pre-laying period to 22 weeks of age. The pre-laying basal diet is shown in Table I. During this pre-laying period, all groups received vit. B_{12}‡ at a level of 12.5 μg per pound of feed. Groups 2 and 4 received terramycin§ at a level of 15 ppm.

At 22 weeks of age when the pullets showed signs of approaching sexual maturity, they were distributed into lots of 16 at random and housed in individual cages with wire floors. Only one group of 16 hens from each of the pre-laying treatments was used in this particular study. The basal diet fed after this time is also shown in Table I. Vit. B_{12} supplementation was withdrawn from Groups 1 and 2, but the terramycin supplementation for Group 2 was continued as before. Records of the age when the first egg was laid, egg production, hatchability, mortality and body weight were kept. The experiment was continued for approximately 10 months. During the latter part of the experiment, eggs were saved from Groups 1, 2, and 4 and were assayed for vit. B_{12} content by a modification of the method of Skeggs *et al.*(5) using *Lactobacillus leichmanii* (ATCC 4797).

TABLE I. Composition of Basal Diets.

Ingredients	Pre-laying, %	Laying, %
Ground yellow corn	24.7	16.6
" barley	15	2.1
" oats	10	2.1
Millrun	10	13.4
Soybean oil meal (solvent)	30	15.9
Dehydrated alfalfa	5	3
Whole wheat	—	40
Ground limestone	2	2.6
Dicalcium phosphate	2	2.9
Salt	1	.6
Riboflavin supplement (500 μg/g)	.3	.6
A and D supplement (4000 I. U. vit. A, 1000 A.O.A.C. units of vit. D/g)	—	.2
$MnSO_4$	8 oz/ton	8 oz/ton

Eggs were saved for hatching and incubated at weekly intervals. The total number of fertile eggs incubated from each group is shown in Table II. Eggs were saved from Groups 1 and 2 for a 15-week period beginning April 24, 1951; eggs from the other 2 groups were saved only during a 4-week period beginning July 13, 1951, since we were primarily interested in determining whether a terramycin supplement would increase the severity of a vit. B_{12} deficiency as suggested by preliminary work with swine.

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‡ Merck vit. B_{12} supplement containing 12.5 mg/lb.

§ Terramycin hydrochloride.

TABLE II. Effect of Terramycin and Vitamin B_{12} on Mortality, Body Weight Gain, Egg Production and Hatchability.

Group No.	Dietary supplements: Pre-laying period: Vit. B_{12}, mμg per lb	Pre-laying period: Terramycin, ppm	Laying period: Vit. B_{12}, mμg per lb	Laying period: Terramycin, ppm	Avg body wt*: Pre-laying period: Initial wt (8 wk), g	Avg body wt*: Pre-laying period: Gain in 14 wk, g	Avg body wt*: Laying period: Initial wt (22 wk), g	Avg body wt*: Laying period: Avg gain, g	Mortality during laying period, %	Avg age at first egg, days	Avg egg production, %	Hatchability: No. fertile eggs incubated	Hatchability: Chicks hatched, %
1	12.5	—	—	—	725	883	1608	490	18.8	177.8	62.5	346	43.1 (20.7–68.9)†
2	12.5	15	—	15	697	881	1578	604	12.5	184.5	60.1	361	78.1 (63.6–100)
3	12.5	—	12.5	—	725	883	1841	446	12.5	179.2	61.6	85	84.7 (78.6–90.9)
4	12.5	15	12.5	15	697	881	1633	527	6.3	183.5	63.3	104	95.2 (94.3–100)

* 90 pullets/treatment during pre-laying period; 16 pullets/treatment during laying period.
† Range of individual hatches.
Difference in hatchability between Groups 3 and 4 was significant at the 5% level.

Results. The results of the experiment are summarized in Table II. Body weight data showed no differences in gain during the developing season. When the initial weights are taken into consideration, it is apparent that the vit. B_{12} and terramycin supplements had no effect on gain during the laying period, either. All 4 groups reached sexual maturity at about the same age. While the data on egg production indicate little or no effect of the terramycin and B_{12} supplements, there appeared to be a greater drop in egg production toward the end of the experiment in the groups not receiving vit. B_{12}. Terramycin markedly increased hatchability, even in the absence of a vit. B_{12} supplement. The vit. B_{12} content of the egg yolk from Groups 1, 2, and 4 was 9.3, 11.83, and 12.2 mμg per gram, respectively. Hatchability of fertile eggs for these groups was 43, 84.7 and 95.2%, respectively.

Discussion. It is difficult to explain the increased hatchability given by the terramycin supplement in Group 2, since no great increase in vit. B_{12} content of the egg was observed. Furthermore, the amount of B_{12} in eggs from the basal treatment should have been sufficient for hatchability, based on the findings of Olcese *et al.*(6) who showed that eggs containing as little as 5 mμg B_{12}/g egg yolk gave satisfactory hatchability. Elam *et al.*(4) using crossbred chicks, showed an effect of penicillin in the reproduction of the fowl in addition to that of vitamin B_{12}. Although the small number of eggs involved does not permit definite conclusions, there was a suggestion in these experiments that the addition of terramycin to a diet already containing vitamin B_{12} further improved hatchability.

Elam *et al.*(4) reported that optimal hatchability occurred when the vit. B_{12} content of the yolk was 3.7 mμg per gram. Olcese *et al.* (6) found that, with a purified diet containing no added vitamin B_{12}, hatchability dropped to 0 after 6 weeks, although the vit. B_{12} of the eggs from these hens contained between 5 and 10 mμg per gram of yolk. The eggs from hens fed a practical diet which supported optimal hatchability also contained between 5 and 10 mμg of vit. B_{12} per gram of yolk. In the present experiment, it was observed that eggs from group 1 hatched poorly

even though the amount of vit. B_{12} in the yolk was greater than that found by Elam *et al.*(4) in eggs that hatched satisfactorily. There is, thus, a contradiction between the vit. B_{12} content of the egg yolk by microbiological analysis and the amount required for hatchability.

Summary. An experiment was conducted with White Leghorn pullets to determine the effect of feeding terramycin alone and in combination with vit. B_{12} on egg production and hatchability. The results obtained indicate that terramycin had little or no effect on egg production and gain in weight. In the absence of vit. B_{12}, hatchability of fertile eggs was definitely improved by feeding terramycin. There was an indication that terramycin fed in combination with vit. B_{12} further improved hatchability.

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Proteinuria in Experimental Renal Hypertension.* (19339)

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Recently the rate of development of proteinuria in three-quarters nephrectomized rats was reported(1). Removal of both adrenals after the proteinuria was well-developed did not significantly alter the rate of protein excretion although Addis and his coworkers have shown that adrenalectomy abolishes the proteinuric action of renin and decreases the rate and amount of excretion of intraperitoneally injected protein. Administration of cortical hormones restores the proteinuric effect of renin and increases the rate and amount of excretion of intraperitoneally injected protein(2-4). The purpose of the present paper is to report on the rate of development of proteinuria in rats made hypertensive by the removal of one kidney and constriction of the other, and its relationship to growth of the remaining renal tissue, development of hypertension and development of renal lesions. Since the renin-angiotonin pressor system is probably involved in the pathogenesis of constrictive renal hypertension and since not only renin but angiotonin as well(2) will induce proteinuria in the rat the possibility exists that the proteinuria of the hypertensive rat is to be explained on this basis. On the other hand the proteinuria might be a consequence of a renal lesion. Study of the time relationships should throw some light on the pathogenesis of the proteinuria.

Methods. 24 male albino rats of the Stanford colony, weighing close to 150 g each at the start of the experiments were divided into 2 equal groups. A base line of protein excretion was established by 4, and a base line of blood pressure by 2, observations. Following one-stage operative procedures on the kidneys these measurements were repeated bi-weekly. In the first group of rats one kidney was removed and the other exposed, handled and replaced. In the second group one kidney was removed and the other constricted by a figure-of-eight silk ligature, according to the technic of Grollman(5). Measurements of protein excretion were carried out by placing the rats in metabolism cages at 4 p.m., col-

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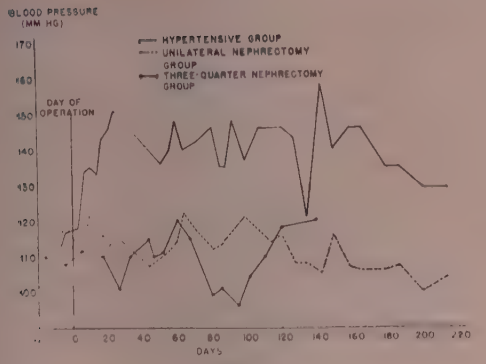

FIG. 1.

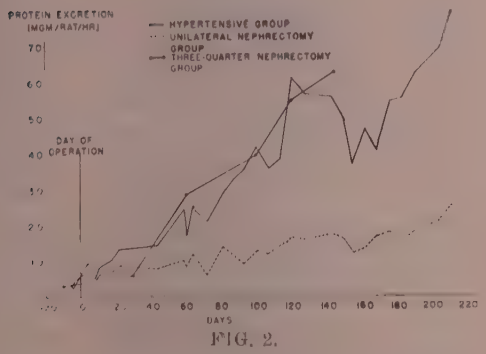

FIG. 2.

lecting for the next 16 hours and analyzing pooled specimens by the Shevky-Stafford technic as described in a previous paper(2). Systolic blood pressure was measured in the tail with the Friedman-Freed microphonic manometer(6) according to a procedure previously described in detail(7). The rats were kept on a stock diet containing 17% protein and 0.3% NaCl. Two of the rats died after unilateral nephrectomy and 3 shortly after renal constriction. The survivors were killed on the 210th day.

Results. Fig. 1 shows the curve of the mean of the systolic blood pressures in the 2 groups of rats. For purposes of comparison the blood pressure mean of a group of three-quarters nephrectomized rats, previously reported(1), is included. The mean pressure rose rapidly and became stabilized after about 3 weeks in the rats with constricted kidneys, while no elevation occurred in the 2 other groups. Expressed as a percentage of the predicted value based on heart weight/body weight tables for this colony(8), the mean heart weight of the hypertensive group was 141 ± 12 (stand. error), of the unilaterally nephrectomized group 106 ± 2, and of the three-quarters nephrectomized group 105 ± 2.4. The corresponding mean weights for kidney tissue were 85 ± 4.4, 81 ± 2, and 70 ± 4.6. Fig. 2 shows the protein excretion expressed as milligrams per rat per hour during the 16-hour collection period. *Renal lesions*: in the rats with constricted kidneys lesions similar to those described in the three-quarters nephrectomized rats were present in variable degree. Intercapillary and extracapillary proliferative and sclerosing alteration with irregular thickening of the basement membrane, irregular distribution of hyaline droplets in the proximal tubule cells and occasionally in the capsular epithelium, were the characteristic features, and did not seem to be related to the constricting band or to the development of pyelonephritis. In a previously studied group of rats with renal constrictive hypertension these lesions were not present nor were they seen in three-quarters nephrectomized rats. Apparently this was because both groups were killed only 50 days after the operative procedures. The delayed development of renal lesions of the type described appears to be characteristic(1,11,12). When hypertension does develop in three-quarters nephrectomized rats it does not manifest itself until a month or more after the operation while in the rat made hypertensive by removal of one kidney and constriction of the other elevation of the blood pressure occurs within a day or two.

Discussion. The development of hypertension and proteinuria under these conditions appear to be independent processes. The blood pressure rises rapidly and levels off while the proteinuria shows a linear increase with time. The proteinuria of the non-hypertensive three-quarters nephrectomized rats roughly parallels that of the hypertensive rats. No relation exists between the hypertrophy of remaining renal tissue and the development of proteinuria. Addis(9) found that by the 20th post-operative day the remaining kidney of the unilaterally nephrectomized rat reached 73% of the predicted 2-kidney weight and for the next 60 days the

weight of the kidney did not change. Constriction of the remaining kidney with a figure-of-eight ligature after contralateral nephrectomy seems to have no adverse effect on its growth although if the contralateral kidney is not removed the constricted kidney is thereafter stunted(7). Addis found(10) that the growth of the stump after three-quarters nephrectomy was rapid, reaching 55% of the predicted 2-kidney weight by the 20th day. Thereafter there was a slow increase in weight of the stump which, however, seems to be dependent on the renal lesion described above. Part of the weight increase is due to distension of the renal tubules with protein-containing fluid.

The immediate loss of renal tissue, drastic though it seems, does not produce pronounced proteinuria. The significance of the renal lesion with regard to the proteinuria is difficult to assess. There is no direct evidence that the lesion is the cause of the proteinuria or that it precedes it. Increased permeability of the glomeruli may be present but cannot be presumed on the basis of the proteinuria alone since the value of the tubular resorptive factor is not known. Measurement of the hemoglobin and creatinine clearances as carried out by Monke and Yuile(13), Lippman, Ureen, and Oliver(14), and Brandt, Frank, and Lichtman(15) might provide data from which to estimate the relative roles of glomerular permeability and tubular resorption. Athrocytic phenomena in the tubule cells probably indicate overloading of a tubular mechanism for absorbing and disposing of protein, but whether this is a consequence of increased filtration of protein cannot be answered at present.

Summary. Rats made hypertensive by the removal of one kidney and constriction of the other show a linear increase in urine protein paralleling the rate and magnitude of increase in urine protein excretion shown by non-hypertensive three-quarters nephrectomized rats. The development of proteinuria is independent of the development of hypertension. No apparent relationship exists between the proteinuria and hypertrophy of remaining kidney. Glomerular and tubular lesions are present in most of the proteinuric rats.

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Production of Meningoceles and Cranioschisis in Chick Embryos with Lead Nitrate.* (19340)

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Since finding abnormal amounts of lead and mercury in some cases of congenital disease of the newborn(1) an attempt was made to induce similar changes in chick embryos with lead, mercury and copper salts.

Various substances introduced into the fertile egg may cause abnormalities in chick embryos. Hammett and Wallace(2) noted that small quantities of lead affected the head and optic anlagen and body growth was retarded. Gray(3) found that lead chloride had an apparent effect on the central nervous system. Later Catizone and Gray(4) noted various central nervous system alterations after the injection of lead chloride, including destruction of the anterior end, failure to close and the production of a "sinuous nervous system". Hansborough(5) reported similar changes in chick embryos following the injection of 2 cc of 1% nicotinic acid. His conclusions were similar to those of Taylor, *et al.* (6) who used pantothenic acid. Franke, *et al.* (7) used selenium to produce monstrosities in chick embryos. Insulin and other chemicals were used by Landauer(8). Hunt(9) noted a variety of abnormal morphological types in chick embryos developing from eggs injected with 2 M sucrose. The principal affected areas were the nervous and circulatory systems. Newcastle virus infection of embryos resulted in defective development of the lens and auditory sac(10).

Procedure. Since the appearance of meningoceles was so striking in the leaded embryos the experiment was confined to observation of this lesion. Occasionally other anomalies such as crossed beaks, absence of eyes or heterotaxy were noted in the uninjected controls, anion controls, copper-or-mercury-treated embryos. However, in no instance was a meningocele seen except in lead-treated embryos. White Leghorn eggs were incubated at 39°C in a moist atmosphere. 0.1 cc of solution was injected into the albumen. Lead nitrate and mercuric bichloride were made up in normal saline to contain 0.5-1% lead or mercury. Copper sulfate containing 1-2% Cu was used. Control eggs were injected with sodium salts of chloride, nitrate and sulfate to test for anion effects.

Results. The results of injections of Pb, Hg and Cu are listed in Table I. A total of 715 eggs was used, of which 78% were

FIG. 1. Meningocele in leaded embryo.

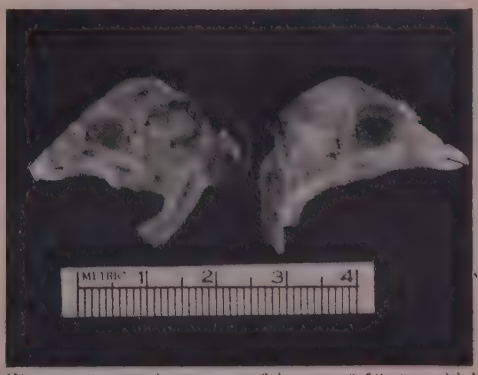

FIG. 2. Sagittal sections of heads of 19 day chick embryos (Fig. 1). Meningocele seen in leaded embryo at left. Saline control at right.

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TABLE I. Survival of Eggs Injected Before Incubation.

Inj. cation, mg	Inj. eggs	Fertile eggs	% dead before 13th day	Incubation day of examination 13	14	15	16	17	18	19	20	21	Alive at termination of exp.	Meningoceles
Pb 1	74	66	79	1/1	1/2	1/1	2/2	4/9		1/1*			7	10
Pb .75	144	109	58	1/4	5/14		1/2		0/1	0/5	3/19	1/3	3	11
Pb .5	24	18	61		0/3				1/4				4	1
Hg 1	60	45	80	1					8				8	0
Hg .75	36	32	53		1				14				12	0
Hg .5	24	15	53								7		7	0
Cu 2	24	19	68		1					2	3		3	0
Cu 1	41	27	74		1				3		3		6	0
Totals	715	344												
% fertile—78														

* Numerator—No. with meningoceles. Denominator—No. examined.

fertile. It is seen that mortality of the chick embryos in the first 13 days of incubation is high and probably is related to toxicity of metallic ions. The meningocele lesion was not grossly apparent until approximately the 13th day of incubation. The eggs were opened from the 17th to the 21st day and no attempt was made to allow hatching. Most of the embryos reaching these days of development were alive, and it can be seen the mortality was extremely high even after the 13th day. The weight of the leaded embryos averaged less than the control embryos. The meningoceles consisted of protrusions of the arachnoid and dura covered by skin. The space was occupied by a thin fluid that appeared to be under slight pressure. These lesions were present in the occipital regions and rarely in the region of the anterior superior portions of the skull. One embryo had 2 such protrusions of the meninges. Lack of bone development in the region of the meningocele was obvious (Fig. 1 and 2). Examination of microscopic sections revealed degenerative changes in the cerebellum and other portions of the brain, due perhaps to pressure from the meningocele and to toxic effect of the lead ion. In experiments not recorded in Table I no meningoceles were seen in embryos from 152 eggs injected with lead ion concentrations of from 0.01 to 0.05%.

Control eggs were inoculated to test effects of the anions used. A total of 185 eggs were injected, each with 0.1 cc of sterile 0.85% NaCl. 81% were fertile and 12% died before the 13th day of incubation. 77% of the embryos survived until the eggs were opened on the 17th to the 20th day of incubation. In most instances development was normal. Rarely was a crossed beak or deformed eye noted. The mean average weight of this group was higher than that of the metal exposed embryos opened on corresponding days. Forty-two eggs, of which 81% were fertile, were injected, each with 0.1 cc of 2% Na_2SO_4. The mortality was 33% in the first 13 days. This was definitely higher than in the saline injected eggs but less than in the metal groups. No gross abnormalities were found in those embryos surviving the 13th day of incubation.

Forty-seven eggs were injected with 0.1 cc of 0.8% $NaNO_3$. 20% of the embryos were dead before the 13th day. Three died on the 15th day and 28 were alive on the 17th day. No gross abnormalities were seen.

Discussion. The production of developmental anomalies by exogenous substances is not a new finding and Mall(11) suggested that most human monsters resulted from such agents. However, during the past decade considerable interest has been stimulated by observations of the relationship of congenital defects and German measles(12). More recently unusually large amounts of lead were found in defective newborn infants(13).

In the present study it is of interest that lead produced grossly defective chick embryos. Copper and mercury did not produce meningoceles although sufficient in quantity to produce death in a similar number of embryos before the 13th day. Eggs from two unrelated flocks of White Leghorns were used and the results were the same. Meningoceles were not present in the non-leaded controls and did

not appear in the copper, mercury or anion injected controls, but appeared in equal numbers in the leaded chicks. It would seem that lead has a specific effect in the production of this particular lesion in the occasional embryo. We do not know if the defect occurs spontaneously. In the human the lesion has been considered to be of hereditary origin since it has appeared in families of the involved infants or embryos(14).

It is quite probable that meningoceles in chick embryos are a late development of the "open nervous system" described by Catizone and Gray(4). They found the lesion most frequently when lead was injected into the subgerminal cavity at 18 hours incubation. Obviously the ion injection in the albumen would be subject to greater variation in its quantitative relationship to the embryo. It is possible that a larger number of meningoceles could have been produced by injection at a different stage of incubation or by exposing the embryo more directly through subgerminal cavity injection. That the lesion could be produced even relatively late in embryonic development is evident from the observation of meningoceles in 3 of 29 embryos which developed in eggs injected with 0.75-1 mg of lead on the 5th day of incubation.

Conclusion. (1) Injection of lead into the albumen of fertile eggs may result in the production of meningoceles in chick embryos. (2) Copper and mercury ions were noted to be as toxic as similar quantities of the lead ion, but meningoceles were not seen in the embryos surviving 13 days incubation. (3) Sodium salts of anion used in the metal experiments failed to produce meningoceles.

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Failure of Nembutal to Block Ovulation in the Hen. (19341)

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Data have recently been accumulated indicating that the nervous system participates in the release of tropic hormones from the pituitary gland(1,2). Specifically it has been reported that the hypothalamus is involved in the release of the luteinizing hormone (LH), thyrotropic hormone (TSH) and adrenocorticotropic hormone (ACTH) from anterior pituitary gland(3-5). Experiments involving electrical stimulation in the rabbit have shown that ovulation occurs much more readily upon stimulation of the hypothalamus than upon direct stimulation of the hypophysis(6,7). Brooks, Beadenkopf, and Bojar(8) reported ovulation in the rabbit following injection of picrotoxin, a central nervous system stimulant. Pre-treatment with phenobarbital prevented the ovulatory-inducing action of the picrotoxin(9) and since barbiturates are known to have a selective action on the hypothalamus this would further tend to involve the diencephalon in the release of LH. Everett and Sawyer(10) have shown in the rat that Nembutal anesthesia between 2 and 4 p.m. on the

day of proestrous will prevent ovulation otherwise expected about 11 hours later. Nembutal is also effective in preventing progesterone-induced ovulation in rats(11).

The present study was undertaken to determine the role of the nervous system in ovulation in the domestic hen. In view of the definite, temporal relationship between LH release, ovulation and oviposition it is possible to calculate the time of LH release from the hypophysis after the time of lay has been determined(12-14). This should make the hen ideal for these studies in that the time of lay of the egg can be recorded without operative procedures. In addition the hen shows an asynchronous rhythm in the time of egg laying in that the first egg is laid in the morning and each succeeding egg of the clutch is laid progressively later in the day until the clutch is ended. The hen then skips a day and starts the next clutch in the morning again. This might indicate that the mechanism is different in this form from other species studied thus far. The present investigation is concerned with the action of Nembutal on normal and progesterone-induced ovulation.

Material and methods. Adult laying hens of the White Leghorn strain were used. The birds were kept in a laying battery under constant conditions of temperature and humidity and supplied with food and water *ad lib.* Artificial lighting was maintained from 8 a.m. to 9 p.m. daily. Records of the time of oviposition were kept for 4 weeks prior to the experiment in order to determine the characteristic, clutch length for the individual hens. Calculations made from these data indicated that LH release was confined to the interval between 10 p.m. and 10 a.m. This was determined on the basis that oviposition in our flock occurred between the hours of 10 a.m. and 4 p.m. and since ovulation precedes oviposition by about 26 hours(12), ovulation must have taken place between 8 a.m. and 2 p.m. Since LH is discharged from 4 to 10 hours prior to ovulation(13,14), the earliest time for LH release would be 10 hours prior to 8 a.m., and the latest time would be 4 hours prior to 2 p.m. Only those hens were used that were expected to release LH at the time of the Nembutal anesthesia. Anesthesia was induced with an initial dose of 70 mg of Nembutal given intraperitoneally. This treatment caused a deep anesthesia in 5 minutes with a duration of one-half to one hour. Prolonged anesthesia was obtained by supplementing the initial treatment with additional injections of 20 to 30 mg of Nembutal every 20 to 30 minutes. The progesterone* was dissolved in sesame oil and injected intramuscularly.

TABLE I. Inability of Nembutal to Block Natural Release of LH in 13 Hens. Anesthesia was maintained for 12 hr (10 P.M. to 10 A.M.).

No. of hens	Time of lay in clutch	No. of hens that laid next egg	% of hens showing apparent release of LH during anesthesia
4	Start	4	100
8	Middle	7	88
1	End (?)	0	0

Results. In a preliminary experiment Nembutal anesthesia was induced in a number of hens at the time calculated for LH release. The anesthesia was maintained for only 2 hours. No interference with ovulation was observed in any of the hens. To remove any doubt as to the failure of Nembutal to prevent ovulation, a second experiment was designed in which the hens were maintained under deep Nembutal anesthesia for 12 hours. A total of 13 hens were used during different stages of their clutch. Anesthesia was started at 10 p.m. and maintained until 10 a.m., the next morning. The occurrence of ovulation was judged by digital palpation through the cloaca and by the time of subsequent lay. Eleven of the 13 treated hens or 85% ovulated indicating a failure of the Nembutal anesthesia to block the normal release of LH (Table I). In one instance the bird may have been at the end of the clutch so that no ovulation could be expected. If this hen is eliminated from the group, then ovulation occurred in 92% of the treated birds.

A third experiment was designed to study the effect of Nembutal anesthesia on pro-

* Progesterone was obtained through the courtesy of Dr. Henderson, Schering Corp., Bloomfield, N. J.

TABLE II. Inability of Nembutal to Block Progesterone Induced Ovulation.

Group	Anesthesia, hr	No. of hens	% ovulating	Mean interval inj. to lay, hr
I	—	12	83	35.1 ± .9*
II	1.5	14	100	35.4 ± 1.3
III	3	5	80	34.4 ± 1.5
IV	6	13	77	35.6 ± 1

* Stand. dev.

gesterone-induced ovulation(15). In all instances 1 mg of progesterone was injected intramuscularly at 11 a.m. on the last day of the clutch. Table II shows the results involving a total of 44 hens, divided into 4 groups. The first group consisted of the control birds and received only progesterone. Groups II, III and IV received progesterone and in addition were kept under anesthesia with successive intraperitoneal injections of Nembutal for 1.5, 3, and 6 hours duration, respectively. The initial injection of Nembutal was made at 10:50 a.m., so that the birds were in deep anesthesia by the time the progesterone injections were made.

Eighty-three per cent of the control group of 12 hens, treated at the end of the clutch, ovulated after the intramuscular injection of 1 mg of progesterone (Table II). However, Nembutal anesthesia for 1.5 to 6 hours failed to block ovulation in the progesterone treated birds. Group II under anesthesia for 1.5 hours showed 100% ovulation, Group III under anesthesia for 3 hours showed 80% ovulation, and Group IV under anesthesia for 6 hours showed 77% ovulation. It is also of interest to note that the mean interval from the time of injection of the progesterone to the time of lay was not affected by the anesthesia (Table II).

Discussion. It has been known for some time that the induction of pseudopregnancy can be inhibited by gaseous anesthetics(16) and barbiturates(17), and it has been suggested that the block occurred in the hypothalamus preventing the release of LTH(17). Recently, the investigations by Everett and his collaborators(10,11) indicating that Nembutal can block ovulation in the rat, led them also to conclude that the hypothalamus was involved in the release of LH. Similar conclusions were arrived at from work on the rabbit(6).

The present results would tend to indicate that the central nervous system may not always be involved in the release of LH in the hen. The failure of Nembutal to inhibit ovulation when anesthesia was maintained for a period of 12 hours should rule out the possibility of miscalculating the time of LH release. In addition, the failure of Nembutal to inhibit progesterone induced ovulation, where the time of LH release is more exact, constitutes added evidence that a stimulus (progesterone) may bring about the release of LH by direct action on the pituitary gland. That the nervous system may impinge on the phenomenon of ovulation is obvious from the influence of light on the time of oviposition but it would appear that in the hens the mechanism is different from that seen in the mammal.

Summary. (1) Nembutal anesthesia was induced for periods of 2 and 12 hours in laying hens in an attempt to inhibit the normal release of LH. Ovulation occurred in more than 80% of the birds despite the length of anesthesia, indicating a release of the luteinizing hormone during the time of narcosis. (2) In a second experiment hens were anesthetized with Nembutal 1.5 to 6 hours and an attempt made to induce ovulation by progesterone. Ovulation was obtained in 100% of the birds anesthetized for 1.5 hours, in 80% of the birds anesthetized for 3 hours, and in 77% of the birds anesthetized for 6 hours. This compares favorably with the 83% ovulation noted in the control hens treated only with progesterone. It may be concluded that Nembutal-induced anesthesia does not interfere with the release of LH in the hen.

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Cytologic Changes in Rat Adenohypophysis Following Administration of Adrenocorticotrophin or Cortisone. (19342)

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An increased pituitary basophile count has been found at post mortem examination of patients dying after receiving pituitary adrenocorticotrophic hormone (ACTH)(1) or cortisone(2). In addition, the hyaline changes described by Crooke(3) were seen in many of the basophile cells after the administration of both hormones(1,4). These changes have usually been considered to be characteristic of Cushing's syndrome. The appearance of these findings following the administration of adrenal cortical hormones strongly supports the theory that the pituitary alterations in Cushing's syndrome are a secondary manifestation of increased adrenal cortical activity. An attempt was therefore made to reproduce these lesions in experimental animals and to define the circumstances under which the changes occur.

Experimental. Male Sprague-Dawley rats were treated with ACTH* or with a microcrystalline suspension of cortisone (Cortone, Merck) according to the schedule in Table I. The daily dose of ACTH was administered subcutaneously in 3 portions—one-fourth of the total at 8:00 A.M. and at noon, and one-half at 5:00 P.M. The phenol control solution was given in a similar manner, in the same volume as the ACTH solution (.025 ml/100 g at 8:00 A.M. and noon; .05 ml/100 g at 5:00 P.M.). A group of normal rats was examined without having been subjected to any trauma. The effects of "stress" were also studied in animals fasted for 15 hours in individual cages in a cold-room at 4°C. At the end of the experimental period, the animals were anesthetized with intraperitoneal pentobarbital. The adrenals were removed, cleaned and weighed on a Roller-Smith torsion balance to the nearest milligram.

The pituitary glands were removed by the dorsal approach. They were fixed in 10% solution of USP formaldehyde, embedded in paraffin and sectioned serially at 5 μ. Multiple sections were stained for differential counting by Mallory's 1% acid fuchsin, orange G, aniline blue stain(5). Selected sections were also stained by a modification of the periodic acid Schiff technic(6). A minimum of 6000 cells of the pars distalis were counted from each gland by the method of Rasmussen(7). In all but two instances, 3 or 4 sections from different areas were included in the count. Two sections from each gland were evaluated for Crooke's hyaline change of the basophile cells. The identity of the slides was unknown at the time of these observations which were repeated on 3 separate occasions.

*Wilson's Corticotrophin, kindly supplied by Dr. David Klein of the Wilson Co.

TABLE I. 4 Rats in Each Experiment.

Procedure	Initial wt (g)	Final wt (g)	Adrenal wt (mg)	% basophiles in ant. pituitary
ACTH, 1 mg/100 g/day, 10 days	227 ± 10	308 ± 19	63 ± 4.6	7 ± 1.2
Cortisone, 3 mg " " 10 "	290 ± 11	233 ± 9*‡	25 ± 1.1*	11.5 ± 1.3‡
Control, .5% phenol, 10 "	229 ± 15	312 ± 14	43 ± 3.6	9.9 ± 1.9†
Normal, no treatment		328 ± 11	48 ± 3.3	5.2 ± .6
Cold-room, 15 hr, fasting		328 ± 13	48 ± 2.2	10.9 ± .8‡

* Significantly different from control ($p < .01$).
† Probably different from normal ($p < .05$).
‡ Significantly different from normal ($p < .05$).

TABLE II. Distribution of Crooke's Change. Two slides were examined for each pituitary on 3 different occasions, and each slide graded from 0 to 4+. Each group consisted of 4 animals.

	Total No. + per group
Normal	7
Phenol control	12
ACTH	63
Cortisone	66
Cold stress	35

Results. The effects of the various treatments on the body weight and pituitary basophile counts are shown in Table I. The effectiveness of the doses of ACTH and cortisone are demonstrated by the increased adrenal weight after ACTH and the decrease after cortisone. The animals receiving cortisone lost weight, whereas all of the other groups gained significant amounts of weight during the experiment.

The administration of cortisone was associated with a significant increase in the pituitary basophile count as compared with the normal rats. No other manipulation significantly affected the proportion of basophiles in the pituitary, except for cold stress, which also raised the basophile count significantly compared to the normal.

The only reliable criterion of Crooke's change was degranulation and the appearance of a deeply cyanophilic homogeneous cytoplasm. This was not necessarily associated with enlargement of the cell. The cytoplasmic masses stained for glyco-protein, as previously described by Laqueur(8). Vacuolization of the cytoplasm, sometimes regarded as a part of Crooke's change(3) was not used as a diagnostic finding as it did not correlate with the cytoplasmic hyalinization.

Discussion. Increased cyanophilia and Crooke's hyaline change have been described in human pituitary glands under conditions known to inhibit the secretion of ACTH. Before the use of ACTH and cortisone in clinical medicine, these changes were seen only in patients with Cushing's syndrome, where the increased blood corticosteroid levels might be expected to inhibit the secretion of ACTH(9). The observation of similar changes in patients receiving ACTH or cortisone appeared to be due to the same mechanism. In our previous communication, we suggested that increased basophilia and Crooke's hyaline change might be morphologic manifestations of storage of ACTH in the pituitary(1).

Differential cell counts were made of the pituitary and a search was made for Crooke's hyaline change in rats under experimental conditions of altered demand for endogenous secretion of ACTH. The release of ACTH was inhibited by the administration of exogenous cortisone or ACTH, and was encouraged by the stress of exposure to cold. Alterations in demand for ACTH were not correlated with changes in the basophile count. Animals which received cortisone or 0.5% phenol, or were exposed to the cold all showed increased basophile counts. The animals which received ACTH, however, showed no significant increase. The lack of response of the pituitary basophiles to ACTH is in accord with the observations of Baker(10), who also failed to find changes in the pituitary basophile count after the administration of this hormone in large quantities.

Marked hyaline change of the basophiles was observed in animals receiving either ACTH or cortisone. Changes of almost equal degree, however, occurred in animals sub-

jected to cold stress. Whereas, the administration of 0.5% phenol caused increased basophilia without Crooke's change, ACTH caused hyalinization without any increase in the basophile count. In the rat, therefore, the two morphologic findings are not correlated. Moreover, Crooke's hyaline change can be found whether the gland is producing increased amounts of ACTH because of stress, or ACTH secretion is depressed by the administration of cortisone. The data indicate that one is unable to deduce the functional state of the rat pituitary gland from the total number of basophiles or the presence of Crooke's hyaline change. These findings are in agreement with the observations of Halmi and Bogdanove (11), who found no correlation between the basophile count and the ACTH content on bioassay of rat pituitaries.

Purves and Griesbach(12) have divided the pituitary basophiles into two groups, "thyrotrophs" and "gonadotrophs", on the basis of response to endocrine manipulation. They showed that the two types of cells have a characteristic anatomic distribution in the pars distalis. In our experiments, the increase in basophiles appeared to involve all areas of the gland. Crooke's change was also noted in all areas of the pars distalis. The morphologic changes which occurred in these experiments did not resemble those described by Purves and Griesbach following administration of estrogens or thyroxine.

It is apparent that our original hypothesis concerning the nature of Crooke's change and increased basophilia is untenable. The fact that the hyaline cytoplasmic masses of Crooke's cells stain for glycoprotein in both the human and rat pituitary is strong evidence that the cytoplasmic substance is not ACTH itself, although it could be a glycoprotein to which ACTH is attached. The purest preparations of ACTH now available do not appear to be a protein nor do they contain carbohydrate (13).

We are unable to relate Crooke's change to alteration of ACTH content of the basophile cells. Possibly the change reflects the storage of a pituitary hormone other than ACTH.

In the human being, prolonged and fatal stress fails to cause the appearance of Crooke's change or of increased basophilia(14). In this respect, therefore, the human differs significantly from the rat. It seems possible that much of the confusion in the literature pertaining to the specific function of the various pituitary cell types may arise from failure to take into account important species differences.

Summary. 1. Adult male rats were treated with adrenocorticotrophic hormone or cortisone, or subjected to stress at 4°C. One control group received 0.5% phenol, and another was untreated. The percentage of pituitary basophile cells was calculated and the basophiles were examined for Crooke's hyaline change. 2. Animals receiving cortisone and those exposed to cold stress were found to have a significant increase in the percentage of pituitary basophiles. The ACTH and 0.5% phenol-treated animals failed to show this response. Crooke's change was prominent in animals receiving ACTH or cortisone and was also seen in the cold stress group but not in either control. 3. In the rat, the percentage of anterior pituitary basophiles and the presence of Crooke's change cannot be correlated with increased or decreased demand for endogenous adrenocorticotrophin. The fact that basophilic granules and the hyaline masses of Crooke's cells stain histochemically for glycoprotein makes it apparent that they are not ACTH. 4. The absence of increased numbers of basophiles or Crooke's change in human pituitaries following prolonged stress points to important species differences in the occurrence of these findings.

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Demonstration of *Escherichia coli* 055 and 0111 Antigens by Means of Hemagglutination Test.* (19343)

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It has been shown by Keogh, North and Warburton(1,2) that certain bacterial antigens may be adsorbed onto red blood cells, thus rendering the erythrocytes agglutinable by the anti-bacterial antibodies. Subsequently this indirect hemagglutination test was used for the demonstration of antibodies against tubercle bacilli (Middlebrook and Dubos)(3), *H. influenzae* type b (Warburton, Keogh and Williams)(4), streptococci (Kirby)(5) and *Pasteurella pestis* (Amies)(6). Furthermore, Hayes and Stanley(7) showed that the somatic antigen (polysaccharide) isolated from an untyped strain of *Escherichia coli* was operative in producing hemagglutination by the homologous bacterial antiserum. The experiments to be described in this communication revealed that the hemagglutination test can be used for the identification of the 0 antigens of two groups of *E. coli*, namely, serogroups 055 and 0111. These antigenic groups of *E. coli* are of particular interest, since they are closely associated with, and, indeed, may be considered as a cause of, epidemic and sporadic diarrheal disease of infants. Furthermore, the observations reported here are of interest in connection with the problem of the nature of the phenomenon of bacterial inagglutinability.

Material and methods. Several strains of *E. coli* serogroups 055 and 0111, isolated from infants suffering from diarrheal disease, were used; in addition, one strain each was kindly supplied by Dr. Joyce Wright, London, England. The organisms were maintained on brain veal agar and transferred weekly. For the hemagglutination test brain heart infusion cultures were used, after the pH had been adjusted to 7.0. Group-specific antisera were prepared in rabbits by injection of suspensions of the organisms; and, for control purposes, *E. coli* 055 B5 and 0111 B4 antisera, made available through the courtesy of Dr. Wm. H. Ewing, Communicable Disease Center, were employed. For the hemagglutination test both human and rabbit red blood cells were washed in physiological saline solution. To the sediment of such a suspension were added *E. coli* infusion broth cultures or filtrates thereof to make a 2.5% red blood suspension. The mixture was kept for 2 hrs at 37°C and stirred repeatedly. The cells were then washed three times with physiological saline solution. The 2.5% cell suspension (vol. 0.25 ml) was added to 0.25 ml of various *E. coli* antisera in serial dilutions and to normal human and rabbit sera, serving as controls. The mixtures were incubated in the waterbath at 37°C and the resulting agglutination was read grossly at various intervals up to 2 hrs.

Results. In the first experiments human red cells were treated with broth cultures of several strains of *E. coli* 0111. Agglutination of these treated cells occurred only irregularly and with relatively concentrated amounts of group-specific antiserum. It was then decided to study the activity of *E. coli* cultures and filtrates which had been heated at 100°C for 2 hrs. The data obtained in one representative experiment, read after 2 hrs. at 37°C. are summarized in Table I.

* Supported in part by a grant from Ciba Pharmaceutical Products, Inc.

TABLE I. Demonstration of *E. coli* 0111 Antigen by Means of Hemagglutination Test. Agglutination by *E. coli* antisera of human red cells treated with heated *E. coli* 0111.*

E. coli antisera	Broth culture	Broth culture filtrate	Washed agar suspension sediment	Agar suspension supernate
		0111 antiserum		
1/100	4	4	4	4
1/200	4	4	3	4
1/400	4	4	1	4
1/800	4	4	—	3
1/1600	—	2	—	2
1/3200	—	—	—	—
0	—	—	—	—
		055 antiserum		
1/100	—	—	—	—

— = No agglutination. 1 to 4 = Various degrees of agglutination.

* Human red blood cells treated with the 4 unheated preparations failed to agglutinate in the above dilutions of antisera. The 055 antiserum strongly agglutinated red cells treated with *E. coli* 055.

It is evident from the data presented in this table that the boiled broth culture, broth culture filtrate, suspension of the organisms and the agar suspension supernate, in contrast to the unheated materials, rendered the red blood cells agglutinable by the *E. coli* 0111 antiserum. That this agglutination is specific for the group-specific antigen 0111† is evident from the fact that several *E. coli* 055 antisera as well as normal sera in like dilutions failed to produce agglutination. Identical results were obtained with red blood cells of both man (blood groups O, A, B, as well as Rh positive and Rh negative) and rabbit.

The specificity of this hemagglutination with *E. coli* 0111 antiserum is substantiated further by the observations that all 4 strains of *E. coli* 0111 rendered the blood cells agglutinable by the 0111 group-specific antisera and that 2 strains of *E. coli* 055 were operative in producing hemagglutination with *E. coli* 055 but not with *E. coli* 0111 antisera. Furthermore, treatment of blood cells with a strain of *E. coli* of a group other than 0111 and 055 (No. 7402) failed to induce hemagglutination by either 055 or 0111 antisera. By means of this indirect hemagglutination test group-specific 0 antigen was demonstrated in 1 to 11 days old infusion broth cultures.

Boiling of *E. coli* 0111 and 055 broth culture filtrates for two hours resulted in greater activity than boiling for one hour only; heating at 100°C for 3 or 4 hours did not substantially increase further the activity of the filtrate. It was found that autoclaving of the filtrates at 15 lbs pressure for 5 and 15 minutes produced active antigenic material, although autoclaved filtrates were somewhat less effective than those boiled for 2 hrs.

Hemagglutination appears within a few minutes after the test is set up and increases in strength during 2 hrs of incubation at 37°C. Centrifugalization at 2,000 r.p.m. for one minute enhances the degree of agglutination. Since it was shown by Wheeler, Luhby and Scholl(8) that trypsinized red blood cells may be agglutinated by incomplete Rh antibodies in saline solution, it was decided to determine whether such cells differ from untreated cells in the above described hemagglutination test. In repeated experiments it was found that trypsinized cells are agglutinated somewhat more strongly than untreated cells and that one-half to one-fourth of the minimal amount of group-specific antiserum causing agglutination of non-trypsinized red blood cells produced specific agglutination of trypsin-treated cells.

Discussion. The above described experiments revealed that both human and rabbit red blood cells may adsorb the somatic antigen of *Escherichia coli* serogroups 055 and 0111 from broth cultures as well as from sterile Seitz filtrates, thus rendering these erythrocytes agglutinable by the respective group-specific antiserum. Since certain bacteria produce agglutination of red blood cells, referred to as bacteriogenic hemagglutination,

† Both Dr. F. Kauffmann, Copenhagen, and Dr. P. R. Edwards, Chamblee, Georgia, who have seen the manuscript, raised the question as to whether or not the antigen demonstrated in the hemagglutination test is the O or the thermolabile B antigen. That the above described test demonstrates the O antigen is evident from the facts that, as shown by Dr. Edwards (personal communication) and in this laboratory, O antiserum gives agglutination to the same degree as serum containing O and B antibodies and that red cells treated with heated broth culture filtrates or agar suspensions engender specific *E. coli* antibodies in rabbits (unpublished data).

the term "indirect bacterial hemagglutination" is suggested for the specific agglutination of red blood cells produced by antibacterial antibodies acting on the antigen adsorbed onto the erythrocytes.

The observation that *E. coli* cultures and their filtrates become highly effective in this indirect hemagglutination test after being boiled for one or two hours in comparison to the unheated specimens is of interest in connection with the phenomenon of bacterial inagglutinability. It has been known for many years that certain strains of enteric bacilli are not agglutinated by the specific O antisera, unless the bacteria are first heated or the agglutination test is carried out at 50°C instead of 37°C. Kauffmann (9) made the important discovery that the heat-labile L or B antigens of *E. coli* are the cause of O inagglutinability. These antigens are not capsular antigens, since, with the exception of a single strain, capsular swelling is not produced by L or B antibodies. Kauffmann calls these antigens surface or envelope antigens, which, because of their location on the surface of the bacterial cell, make it impossible for the O antibody to reach the O antigen. Inactivation of the L or B antigens by boiling of the bacterial suspension exposes the O antigen to its homologous antibody. The observation that boiling of the sterile *E. coli* broth filtrate also renders the filtrate active in the above described indirect bacterial hemagglutination test for the demonstration of the O antigen suggests that the interference of the L or B antigens with O agglutination or hemagglutination is not necessarily dependent on the topographic arrangement of these antigens inside the bacterial cell. Rather, one may consider the possibility that L or B and O antigens may be present in the intact bacterial cell as well as in culture filtrates as a complex and that the L or B antigens block the reactive group or groups of the O antigen.

The question arises as to whether or not adsorption of bacterial antigens onto red blood cells occurs also *in vivo*. If this be so, one may be able to demonstrate in various infections red blood cells which are specifically agglutinable by the homologous antibacterial antiserum. Furthermore, it is conceivable that in such patients hemolysis and anemia may follow the development of bacterial antibodies. Such an occurrence of events would represent indirect bacterial isohemolysis, since the patient's red blood cells are being agglutinated and/or hemolyzed by his own antibodies and the antibodies do not react with red blood cell antigens but rather with bacterial antigens. Studies are now in progress to elucidate these moot questions.

Summary. (1) Boiled broth cultures and sterile Seitz filtrates thereof of *Escherichia coli* serogroups 055 and 0111 render red blood cells of man and rabbit specifically agglutinable by the homologous group-specific antisera. Unheated cultures and filtrates are only slightly effective. Trypsinized red blood cells are somewhat better agglutinated than non-trypsinized cells. (2) The significance of the findings regarding the indirect bacterial hemagglutination test is discussed with particular reference to the phenomenon of bacterial inagglutinability.

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Influence of Cortisone on Natural Course of Malaria in the Pigeon.*(19344)

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Experimental infections of tuberculosis in rabbits(1), guinea pigs(2), rats(3), and mice (4) have been found to react adversely to treatment with cortisone. Enhancement of the bacteremia(5) and earlier death of mice infected with Group A streptococci and of albino rats infected with Type I pneumococci have been reported. In the blood of mice experimentally infected with *Trypanosoma cruzi*(6) the number of parasites was increased three to fourfold by cortisone. The extent of cutaneous infections with *Trichophyton metagrophytes,* vaccinia virus and *Staphylococcus aureus*(7) has been increased, but skin sensitivity was not affected by cortisone treatment. Schmidt and Squires(8) have just reported the results of studies on malaria infections in monkeys treated wtih cortisone. No significant effect was noted on the parasite count previous to the crisis. Following the crisis the parasitemia persisted for a much longer period of time, but at a parasite level well below the initial peak count.

Attempts to determine the reactions of the hosts tissues to the administration of cortisone have revealed an interference in the production of granulation tissue in wounds of rabbits(9), a reduction in spleen size in mice(10), inhibition of tissue reactions to bacterial filtrates(11), and depression of the total cellular response to intraperitoneal injections of sterile mineral oil(12). Results of cortisone treatment in many kinds of experimental infections and the increased activity of certain macrophages produced by adrenal cortical extract reported by Gordon and Katsh(13) suggested studies on the action of cortisone on malaria infections and on immunity to reinfection. This report is concerned with the host-parasite relationship in pigeons infected with malaria and treated with cortisone.

Materials and methods. Infections of the 1P strain of *Plasmodium relictum* in pigeons were treated with cortisone (cortone acetate, Merck) in doses of 5 mg daily injected intramuscularly. The pigeons were inoculated by intravenous injection of blood from an infected bird. A comparable number of parasites was inoculated into each bird. Cortisone dosage was initiated 2 or 3 days before infection, or during the decline or at the termination of parasitemia. In one experiment in which the effectiveness of paludrine on the cortisone treated infections was studied the paludrine was administered by oesophageal tube in daily doses of 2 mg, the minimal effective dose. Thin blood films were made daily and stained by the Giemsa method. The results are recorded as the number of parasites per 10,000 R.B.C.

Results. Eleven pigeons have been given cortisone beginning treatment one to 3 days prior to inoculation. In 8 of these birds the initial peak infection was within the "normal" range and in the other 3 the peak was considerably higher than in any of the infected controls (Fig. 1).

The peak in the parasite number occurred at approximately the same time as observed for untreated infections. The decline in the parasitemia parallels that of the untreated infections for the first 2 or 3 days after which the parasite number abruptly begins a marked increase. The birds frequently die at this stage of infection. In untreated birds the parasite decline continues until none are found by the 9th or 10th day.

When cortisone treatment was withheld until 2 or 3 days following the peak parasite number the same effect was noted on the parasitemia. The number of parasites increased immediately and in most cases remained high until the bird died. Few of the pigeons were able to survive the period of prolonged high parasitemia. One bird of this series was able to reduce the infection following the second

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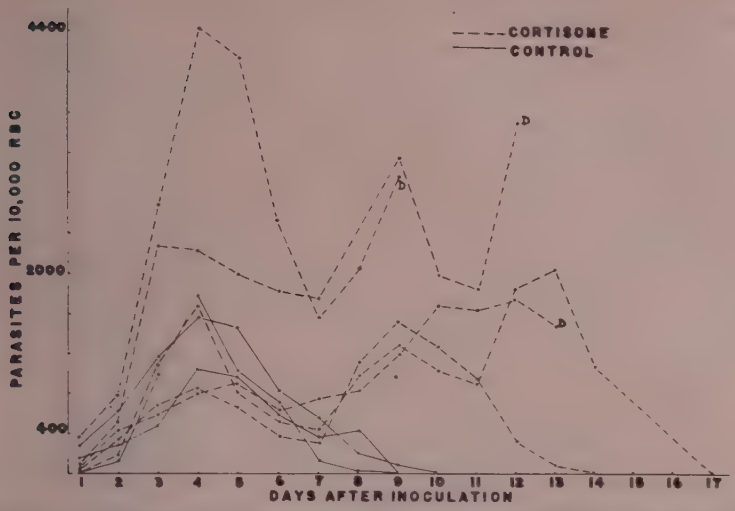

FIG. 1. Effect of cortisone on the parasitemia of pigeons infected with *P. relictum*. Cortisone dosage initiated 2 or 3 days before inoculation. D — bird died.

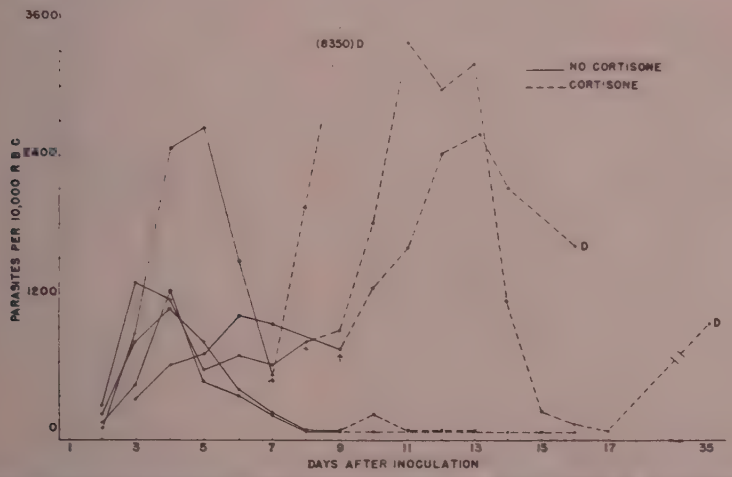

FIG. 2. Effect of beginning cortisone administration after the initial peak in parasitemia. Arrows indicate parasitemia on day of initial cortisone dosage. D — bird died.

peak to almost no parasites but continued cortisone dosage produced a third increase which resulted in death on day 35 of the infection. Cortisone had been reduced to 2 mg daily on day 26 and terminated on day 30. The infection was still on the increase at the time of death (Fig. 2).

In order to determine whether or not cortisone has a direct stimulating effect on the parasites, paludrine in 2 mg doses daily (minimal effective dose) was administered to 3 cortisone-treated birds. The infections were controlled with only an occasional parasite being found.

Daily injections of cortisone into 2 birds with latent infections failed to produce relapse. The physical condition of these birds appeared not to be affected. They remained healthy during the treatment in contrast to the cortisone treated birds with patent infections. Three birds with latent infections were given heavy superinfecting injections of para-

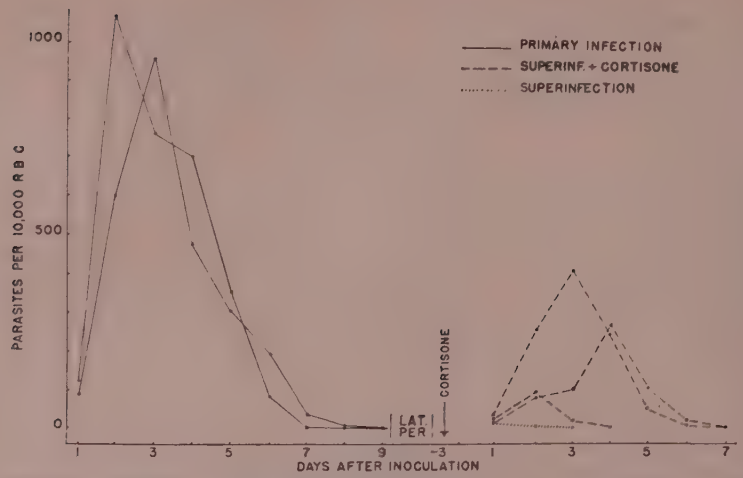

FIG. 3. Effect of cortisone on superinfections. Cortisone dosage was initiated 3 days before superinfection.

sites on the third day of cortisone therapy. The parasites increased in number attaining a peak count of approximately 30% of the initial peak infection on the 3rd or 4th day. A few parasites persisted through the 6th and 7th days (Fig. 3).

Discussion. The results of treating pigeons infected with *P. relictum* with cortisone suggests an interference with the action of the immune mechanism. The development of the immunity apparently is stopped short of full effectiveness before the parasites are reduced to the latent condition. The large number of parasites destroyed following the peak infection may act as somewhat of a blockade of the phagocytes of an impaired system giving the parasites a renewed opportunity to increase and accumulate in the blood. The effectiveness of the entire immune mechanism may be reduced, or some one or more factors of its system prevented from completing its function. Once the immunity is established and the infection assumes the latent condition cortisone apparently is without significant effect. A slight impairment in the function of the immune mechanism appears to result from the cortisone when a heavy inoculum of highly infected blood is injected. There is no indication that the increase in the parasite number in these birds is due to the action of the cortisone on the parasites. This strain of malaria in the pigeon has never been observed to relapse. With other strains which have a tendency to relapse it would appear that cortisone could possibly reduce the protective mechanism of the host to the extent that the infection would relapse. In monkeys infected with *P. cynomolgi* in which relapses are frequent Schmidt and Squires(8) have found that cortisone increases the number and intensity of relapse. Further studies are being made in an attempt to elucidate the mechanism of action of cortisone on humoral and cellular factors which may be concerned with resistance to malaria.

Summary. Cortisone has been found to increase markedly the parasitemia in pigeons infected with *P. relictum.* The increase is most apparent following the primary crisis. In this strain of malaria in which no relapse occurs normally cortisone was without effect on the latent infection.

The technical assistance of Mr. Paul Hudgins is gratefully acknowledged.

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Pharmacologic Dissociation of Behavior and EEG "Sleep Patterns" in Dogs: Morphine, N-Allylnormorphine, and Atropine. (19345)

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Clinical experience has demonstrated that alterations in the state of consciousness are usually associated with changes in the electroencephalogram(1). In particular, characteristic changes in the electroencephalogram occur during natural sleep or after the administration of certain hypnotic drugs, notably the barbiturates(2,3). However, there is evidence that the mechanisms which subserve sleep may be dissociated from those which subserve synchronization and desynchronization of the electroencephalogram. Thus, Magoun(4) has shown that after destruction of the posterior hypothalamus in the cat, afferent sensory impulses may produce "arousal" patterns in the electroencephalogram, while the animal remains quite somnolent. The reverse has also been noted in a few instances. Thus, Andrews(5) observed that in one human subject, a single "analgetic" dose of morphine produced "sleep waves" in the EEG while the patient was wide awake. Cahen and Wikler(6) noted that in the rat, morphine produced "burst" and slow activity in the EEG, although the animal had to be restrained to permit recording. In this species, the "burst and slow wave" pattern produced by morphine was indistinguishable from that produced by pentobarbital, although in the latter case, the animal was anesthetized.

The purpose of the present report is to demonstrate that such dissociations between "sleep patterns" in the EEG and behavior can be produced regularly by administration of a number of drugs under certain experimental conditions, and to discuss the significance of these phenomena in relation to the mechanisms of sleep and the regulation of spontaneous rhythmic cortical electrical activity.

Methods. Twenty-three experiments were made on 12 mongrel adult dogs, in which permanent "mercury-platinum cup" dural electrodes had been implanted in the anterior, middle and posterior portions of the skull (corresponding roughly to the "frontal," "parietal" and "occipital" regions of the cerebral cortex) on both sides of the head symmetrically, according to the method of Wikler and Altschul(7). For recording, the animals were trained to lie quietly on a table in an electrically shielded room, without anesthesia or curarization. "Unipolar" tracings were obtained by pairing leads from each mercury-platinum electrode with a needle electrode inserted in the ipsilateral ear. Various "bipolar" tracings were also made. An eight-channel Grass ink-writing electroencephalographic apparatus was employed throughout.

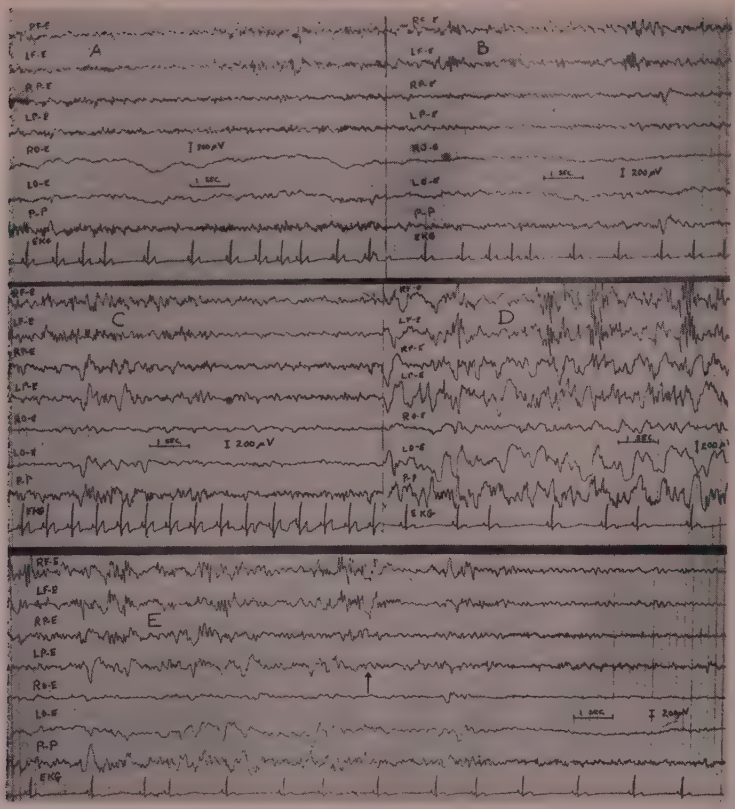

FIG. 1. Dog RCP-IC-1. Effects of "sedative" dose of morphine on the electroencephalogram. A, control. Note periodic "bursts" in frontal tracings. B, control at lower gain. C, 11 min after subcutaneous injection of morphine (approx. 5 mg/kg). Note admixture of random moderate to high voltage slow waves. Dog restless, vomited shortly before sample. D, 23 min after morphine. Note irregular high voltage slow activity in all tracings, and bursts of high voltage 12 cps. rhythms in frontal tracings. Dog apparently awake, eyes open, though dozing occasionally. E, 27 min after morphine, behavior same as in D. At arrow, dog alerted when called softly. Note sudden transformation of EEG from "sleep" to "waking" pattern.

All drugs were prepared in aqueous solutions and were administered subcutaneously in the following dose ranges: morphine 5-150 mg/kg, N-allylnormorphine 2.5-30 mg/kg, and atropine 2-8 mg/kg. An observer was always present in the electroencephalographic chamber and observations on behavior and the EEG were made simultaneously at frequent intervals. For present purposes, only the events which occurred early (*i.e.*, within 15-30 minutes) after injection of the drug will be described, since it was during this period that "sleep patterns" in the EEG were consistently recorded in each case.

Results. In most experiments, the control electroencephalographic patterns were characterized by a predominance of "low voltage fast" activity in all tracings, with an admixture of slower random or rhythmic activity in the anterior tracings. The latter were more prominent when the animal appeared to be "relaxed." Occasional "bursts" of high voltage rhythmic 8-14 cps. spike-like discharges also appeared in the anterior tracings when the dogs appeared to be dozing naturally, or in other experiments, after sleep was induced by intravenous injection of pentobarbital (approximately 15 mg/kg).

Within 15-30 minutes after injection of morphine, N-allylnormorphine, or atropine, the EEG exhibited marked changes. Regardless of the nature of the drug administered,

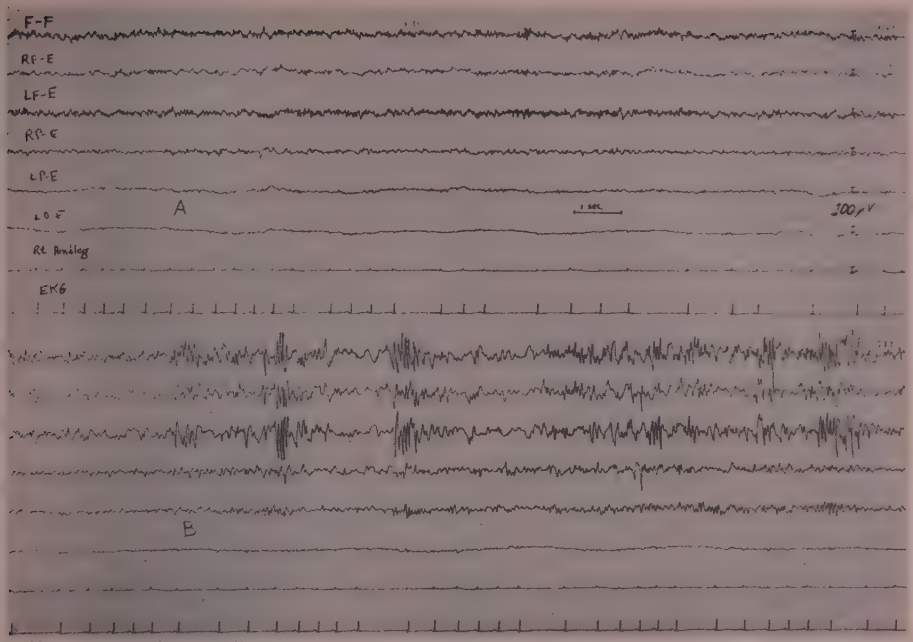

FIG. 2. Dog IC-16. Effects of N-allylnormorphine on the electroencephalogram. A, control. Dog awake. Note predominantly non-rhythmic fast activity in EEG. B, 12 min after subcutaneous injection of 2.5 mg/kg of N-allylnormorphine. Dog alert, moves eyes spontaneously and in following objects. Note typical bursts and slow wave "sleep" pattern in frontal EEG tracings and moderate diffuse slowing in parietal tracings.

the electroencephalographic pattern resembled strikingly that which was observed when the animal appeared to be asleep prior to medication, and consisted of alternate bursts of high voltage 8-12 cps. rhythmic "spike-like" discharges and random or rhythmic high voltage slow (2-6 cps.) waves. These were most prominent in the anterior tracings and were generally bilaterally symmetrical and synchronous. A variable amount of slow activity was also observed in the middle and posterior tracings.

Concomitantly, however, marked differences were observed in the animals' behavior. After the smaller doses of morphine (5-10 mg/kg), the dogs appeared to be dozing, but responded instantly to mild stimuli, such as whistling or calling by name. In these cases, alterations in behavior and EEG pattern appeared simultaneously (Fig. 1). After administration of larger doses of morphine, "arousal" reactions, both with respect to behavior and the EEG pattern, were more difficult to achieve, although the EEG pattern was indistinguishable from that which was observed after the smaller doses.

After administration of small doses of N-allylnormorphine (2.5 mg/kg), the dogs appeared to be at least as alert as before medication and followed objects with their eyes, although concomitantly "sleep patterns" were recorded in the EEG (Fig. 2). With larger doses, mild sedation occurred, but the animal continued to follow objects and respond to mild stimuli.

Following injection of atropine, the dogs were definitely "excited" and had to be restrained to permit recording, at a time when "sleep patterns" were evident in the EEG tracings (Fig. 3). When released, these atropinized animals jumped off the table and spontaneously returned to the animal quarters in the laboratory. Later, periods of excitement alternated with periods of sedation or apparent sleep, yet the "burst-slow wave" pattern in the EEG continued unchanged throughout.

Discussion. These findings have added sig-

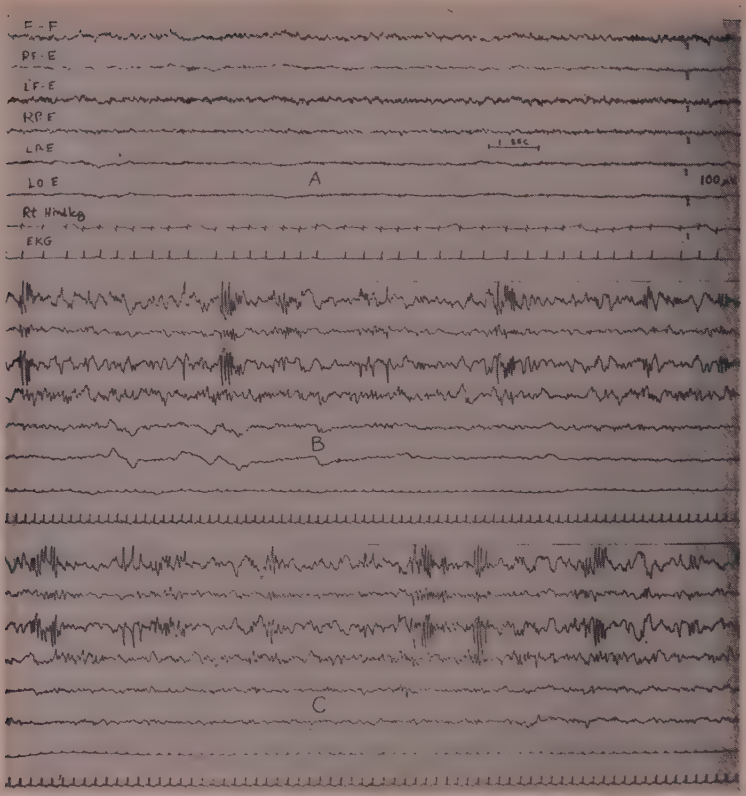

FIG. 3. Dog IC-20. Effects of atropine on the electroencephalogram. A, control. Dog awake. Note predominantly non-rhythmic fast activity in EEG. B, 23 min after subcutaneous injection of atropine 7.2 mg/kg. Dog excited, trying to right self, whining. Note concomitant burst-slow wave "sleep" pattern in EEG. C, 31 min after atropine. Dog quiet, eyes closed, apparently asleep. Note EEG pattern same as in B. This pattern continued unchanged for several hours after atropine while periods of excitement alternated with periods of sedation.

nificance when they are compared with the effects of pentobarbital. In amounts sufficient to produce "burst-slow wave" patterns in the EEG, this barbiturate produces a marked degree of unresponsiveness in both the rat(6) and the dog(8). It is evident, therefore, that the mechanisms which subserve "sleep" and those which subserve the "burst-slow wave" EEG patterns are distinct from each other, although they are very often closely interlocked. Furthermore, one cannot state that "sleep" causes the "burst-slow wave" patterns, or vice-versa. Rather, it appears that they are often concomitant phenomena and that it is necessary to investigate further the precise conditions under which they can be expected to occur simultaneously.

Direct neurophysiological evidence exists which indicates that the mechanisms which regulate synchronization and desynchronization of the spontaneous electrical activity of the cerebral cortex are subserved by neuronal systems which are independent of those which subserve the transmission of sensory impulses to cortex via relay nuclei in the thalamus(9). The pharmacological data here presented suggest that such synchronizing and desynchronizing mechanisms are also independent of those mechanisms which subserve consciousness. Furthermore, the fact that pentobarbital, morphine, N-allylnormorphine, and atropine can produce very similar EEG changes but quite diverse changes in many aspects of behavior(10), suggests that the spontaneous

electrical activity of the cerebral cortex reflects the activity of neuronal systems which, in part at least, are independent of those neuronal systems that subserve behavior in general. The present studies cast no light on what the functions of such hypothesized independent neuronal systems might be, but other data indicate that they may be related to cerebral (not necessarily organismal) homeostasis(11).

Summary. 1. In unanesthetized, uncurarized dogs certain dose of morphine, N-allylnormorphine or atropine produce similar changes in the EEG, which are identical with, or resemble closely, the "burst-slow wave" patterns which occur in natural sleep or during pentobarbital anesthesia. 2. The synchronizing mechanisms which regulate the electroencephalogram are distinct from those which regulate the state of consciousness, although frequently, they are functionally interlocked. 3. The significance of these findings is discussed with reference to the possible functions of the spontaneous electrical activity of the cerebral cortex.

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Chemically Induced Degeneration of Chick Mesonephros.* (19346)

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Considerable attention has been given to the study of the effect of chemical agents on the development of chick embryos. Various workers have treated chick embryos with substances such as insulin(1-4), nicotinic acid (5), lead(6-9), and sucrose(10) and observed a variety of abnormal morphological types.

During recent experiments on the toxicity of various chemicals on chick embryos, it was observed that injection of tantalum hydroxide induced hemorrhage in the capillaries of the chorioallantois membranes and in the kidney (11). The kidney was enlarged and congested with blood and lymph. This observation prompted a histological study of kidneys of chick embryos of various ages and of day-old chicks following injection of various substances.

Materials and methods. The eggs for this investigation were from Rhode Island Red hens at the Agricultural Research Center, Beltsville, Md. The eggs were placed in forced-draft incubators on the day laid and incubated from 10 to 18 days before injection. The following substances were injected: DL-alanine, anthranilic acid, 3-hydroxyanthranilic acid, and salts of tantalum, thorium, and lanthanum (Table I). Distilled water was injected into the eggs employed as controls. The procedure was to inject distilled water or chemical in aqueous solution, not exceeding 0.5 ml in volume, into the allantoic cavity. The opening was then sealed with melted paraffin. Approximately 30 eggs were injected with various chemicals or distilled water at

* This project was supported in part by the U. S. Atomic Energy Commission.

TABLE I. Effect of Injections of Various Chemicals on Chick Mesonephros.

Inj. material	Quantity inj.	Day of incubation — Inj.	Day of incubation — Examined	Appearance of organ — Macroscopic	Appearance of organ — Microscopic
Distilled water	.5 ml	10	12	Normal	Normal
	.5	15	18	"	"
	.5	18	1 day chick	"	"
	.5	1 day chick	6 hr later*	"	"
Tantalum hydroxide	25 mg	10	12	Hypertrophic and hemorrhagic degenerate	
	25	15	18	" " "	"
	25	18	1 day chick	Normal	—
	25	1 day chick	6 hr later*	"	Normal
Lanthanum chloride	20 mg	10	12	Hypertrophic and hemorrhagic degenerate	
	20	15	18	" " "	"
	20	18	1 day chick	Normal	—
	20	1 day chick	6 hr later*	"	Normal
Thorium chloride	50 mg	10	12	Hypertrophic and hemorrhagic degenerate	
	50	15	18	" " "	"
	50	18	1 day chick	Normal	—
	50	1 day chick	6 hr later*	"	Normal
DL-alanine	50 mg	10	12	Hypertrophic and hemorrhagic degenerate	
	50	15	18	" " "	"
	50	18	1 day chick	Normal	—
	50	1 day chick	6 hr later*	"	Normal
Anthranilic acid	20 mg	10	12	Hypertrophic and hemorrhagic degenerate	
	20	15	18	" " "	"
	20	18	1 day chick	Normal	—
	20	1 day chick	6 hr later*	"	Normal
3-hydroxy-anthranilic acid	6 mg	10	12	Hypertrophic and hemorrhagic degenerate	
	6	15	18	" " "	"
	6	18	1 day chick	Normal	—
	6	1 day chick	6 hr later*	"	Normal

* Examined every half day until 7 days of age, then at 14 days of age.

each stage of development. They were broken and the embryonic kidneys removed, fixed in Bouin's, sectioned, and stained with Delafield's hematoxylin and eosin. Microscopic examination was then made to determine the extent of damage.

Results. The results, summarized in Table I as well as illustrated by microphotographs in Plate 1, reveal extensive kidney damage in the younger embryos following injection of the various chemicals. Histological examination of eggs injected on the 10th and 15th days of incubation revealed severe damage to the mesonephros (Fig. 1).† The damage was characterized by massive hemorrhages and marked edema throughout the intertubular tissue. Blood cells and edema fluid replaced large areas of the parenchymal tissue. Many of the tubules and glomeruli disintegrated or disappeared entirely, while in others edema fluid was noted between the intact membrane and the lining cells. In some of the tubules the cells were in a jumbled mass with an intact basement membrane. The primary pathological alternations were degeneration and alteration of many of the tubules and glomeruli as a result of the pressure exerted by edema fluid and the extravasated blood cells.

Chicks hatched from the eggs injected on the 18th day of incubation showed no evidence of kidney damage. The same was true of chicks injected 1 day after hatching and examined 24 hours, 7 days, and 14 days later (Fig. 3). No damage was visible at any time in kidneys from embryos and chicks injected with distilled water (Fig. 2 and 4).

Since kidney damage occurred in embryos from eggs injected on the 10th and 15th days of incubation and no damage occurred in embryos from eggs injected on the 18th day of incubation, and as day-old chicks, it is evident that the damage must be associated with the development of the embryonic kid-

† Acknowledgment is made to Dr. William T. Shalkop for his assistance in evaluating the damage to the mesonephros.

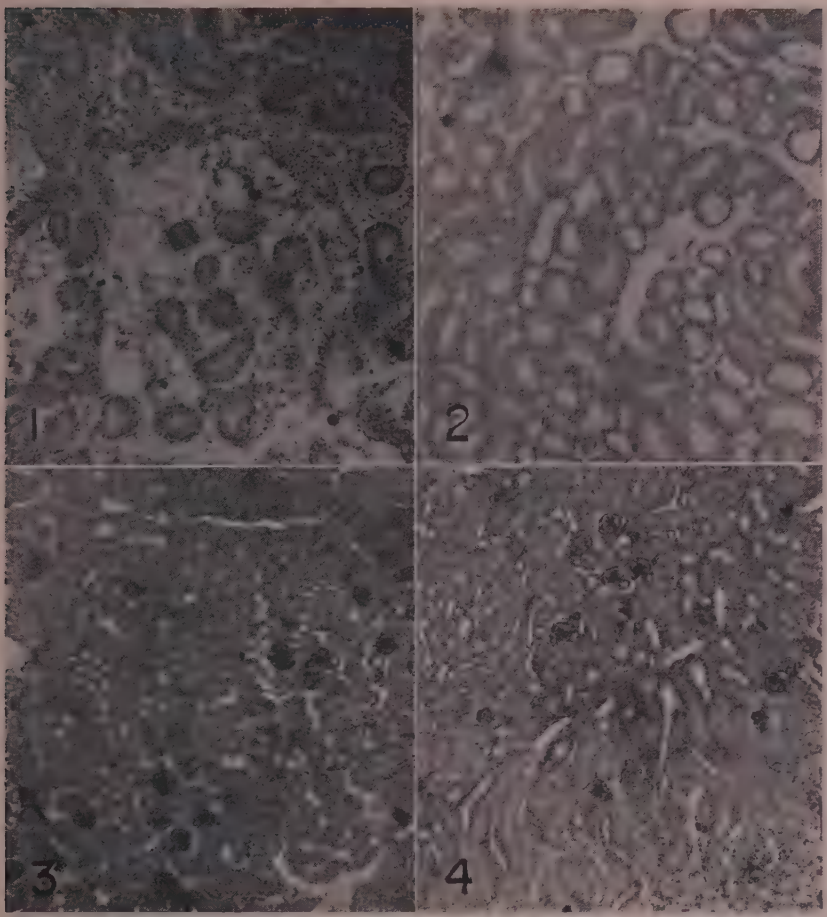

FIG. 1. Median cross section of mesonephros from 10-day embryo injected with tantalum hydroxide and examined 24 hours later. Note evidence of hemorrhage and edema as well as degenerative changes. × 150.

FIG. 2. Median cross section of mesonephros from 10-day embryo injected with distilled water as injected control. Tissue appeared normal. × 150.

FIG. 3. Median cross section of metanephros from day-old chick injected with tantalum hydroxide and examined 24 hours later. Tissue undamaged. × 150.

FIG. 4. Median cross section of metanephros of day-old chick injected with distilled water as injected control. Tissue appeared normal. × 150.

ney. Patten(12) and Lillie(13) state that after the 11th day the developing metanephros begins to become active and the mesonephros degenerates. In this investigation it was noted that in some cases histologically normal mesonephros was present with histologically normal metanephros in the same embryo from eggs that had been incubated as long as 17 days. Needham(14) reports that by the 20th day the mesonephros has atrophied until it is small, thin, and pale. From the evidence presented it appears that the chemical agents injected damaged mesonephric tissue but not metanephric tissue.

Summary. In an experiment on the toxicity of various chemicals on developing chick embryos it was demonstrated that the kidneys of 10- and 15-day embryos were severely damaged by injections of various chemicals. The kidneys of older embryos, those of day-old chicks, and those of control embryos of various ages treated with distilled water gave no evi-

dence of kidney degeneration. From the experiment it appears that the chemical substances injected had a toxic effect on the mesonephros but not on the metanephros.

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Effect of Ploidy in Photoreactivation.* (19347)

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This article will present and discuss some preliminary results of measurements of the relations between ultraviolet dose and survival of haploid and diploid strains of a common species of yeast, both in the dark, and with subsequent irradiation by visible light following initial ultraviolet irradiation. While much work on the effect of ultraviolet light on survival of microorganisms has been published (1), only very limited quantitative studies have been made on the yeast cell in particular; one of the purposes of this work is to emphasize the advantages of the yeast as a test organism in the study of radiation damage. That is, there are available strains differing only in ploidy,‡ making studies on genetic effects relatively easy. The cells are large (5 μ diameter for diploid), easily grown, and give rise to several mutant strains, which are often colored. The present work gives survival curves of the diploid and haploid strains of *Saccharomyces cerevisiae* (SC6 and 7 respectively) both with and without subsequent photoreactivation, with a view to testing the Novick and Szilard theory of the reactivation phenomenon(2).

Procedure. For the initial radiation, 10 ml suspensions of yeast cells in sterile distilled water contained in 50 ml short beakers, of concentrations convenient for plating, were placed on a Gump shaker about 50 cm below the center of a G.E. Sterilamp; after doses up to 220 sec. exposure, aliquots were taken and spread on the surfaces of the growth medium (Difco potato-dextrose agar) in petri dishes. The remainder of the irradiated batch was then used for photoreactivation. The visible light source consisted of four photoflood lamps, arranged such that the light intensity, as measured with a photometer, was uniform over a large area of a transparent plastic shelf supported about 20 cm above the lamps; a glass tray containing 0.05 M $CuSO_4$, interposed between the yeast suspensions and the lamps, effectively kept the temperature rise at the position of the yeast less than 3°C/hr. The concentration of the suspensions varied from 3×10^3 to 5×10^5 cells/ml; within these limits the survival for a given dose did not, as shown by auxiliary experiments, depend on

* This research was partially supported by U. S. Atomic Energy Commission and carried out in the laboratory of Professor R. E. Zirkle.

† Post-Doctoral Research Fellow of the National Cancer Institute.

‡ Genetic structure of yeast is described at length in C. C. Lindegren, Genetics and Cytology of the Yeast Cell (Carbondale, 1950).

the concentration. Greater concentrations resulted in too strong absorption of the radiation. The survival probability was then obtained in the usual way, as the ratio of survivors on a plate poured from an irradiated sample, to those poured from the control sample. However, several complicating factors make it necessary to consider the criterion for the death of a yeast. Some of these complications are not so apparent in the case of *E. coli,* for example. Thus, the yeast cells may sporulate; they can metabolize quite actively, even though unable to divide; storage of essential nutrients can strongly affect the division rate, even to the extent(2) that cells may lie dormant (glycogen storage) in a sated condition, if healthy, or furnish enough material to produce as many as, say, 10 daughters from a severely damaged parent. Further, since "death" due to ultraviolet irradiation is apparently not a well defined state, some cells, which might eventually divide but whose division has been delayed greatly, might, because of depletion of the medium by the cells with shorter delays, never appear as viable. Finally, as in any experiment with U.V.(3) there is generally a large spread in radiation resistance in the original population due to a spread in the ages of individual cells, in physiological state, and even, apparently, in strain, *i.e.,* cells apparently alike in every other way (age, state) may still differ strongly in their resistance to the radiation. Therefore, prior to irradiation, the cells were transferred from slant cultures on PD agar to a medium containing only inorganic salts(4), and left for 36 hours, while the air was bubbled through; just before the run, this suspension was centrifuged, the cells washed in isotonic salt solution, resuspended in sterile water and filtered through 2 layers of sterile absorbent cotton. Microscopic examination showed the absence of any significant number of clumps of cells, and by comparison of the actual microscopic count and the colony count, all cells taken from the fern spore medium were viable. After this treatment, cells in a sample of this suspension should be of about the same age and physiological state, with a small amount of stored material.§ The last point was checked, qualitatively, by comparing the survival obtained by microscope count (scoring microcolonies of n cells or more as produced by "living" cells, of less than n cells as "dead") with the survival obtained from colony count in a petri dish. Without the above pretreatment of the cells the "microscopic" survival was much greater than the "macroscopic" unless $n \sim 10$; with the treatment, the two counts agreed fairly well with $n = 3$. Further, to check on the effect of the growth medium, aliquots of suspensions irradiated to about 10%, and 1%, survival were spread on 4 pairs of plates containing (a) standard PD agar, (b) PD agar enriched with 2% yeast extract, (c) PD agar and 2% casein hydrolysate, (d) PD agar on which about 300 large colonies had grown, the colonies removed, the agar washed in distilled water and then resterilized. No significant difference in survival was observed between any of these, although a difference in delay (time to reach full growth) was apparent, and the average size of individual colonies differed somewhat from one pair to another. All yeast stocks were subcultured every 48 hours on agar slants, and to check that the haploid had not become contaminated, single colony isolates, taken from the survivors of an X-ray irradiation, were grown to large cultures and samples again X-irradiated. Since the exponential curve, with the LD50 measured by Zirkle and Tobias(5) was obtained, it was assumed that this was indeed the SC7 strain; the average U.V. curves obtained with this material agreed with the average curve on the stock material. Runs made just as above but with the haploid cells taken from 6- to 8-day-old cultures often showed a greatly increased resistance in the haploid, with the semilog curve sometimes concave upwards. Presumably this was due to contamination of the haploid stock with diploid because of the tendency for the haploid to undergo illegitimate conjugation (this was a complicating factor in the work of Zirkle and Tobias).

§ Work by T. H. Wood, to be published, indicates that this procedure should be used with caution, if at all, if the aim is to obtain a homogeneous population. However, while the criticism is valid, the essential results of this work are probably correct.

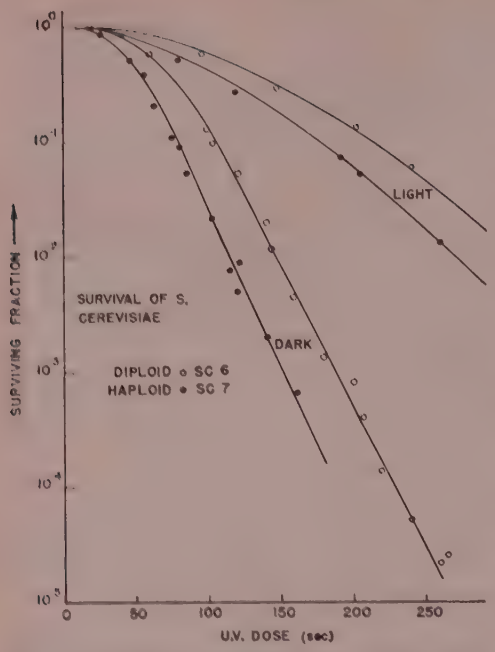

FIG. 1. Survival of diploid and haploid yeast under the action of ultraviolet light ("dark") and with irradiation by visible light subsequent to the initial irradiation by U.V. ("light").

Fresh cultures showed no such enhanced resistance, and the diploid runs were quite reproducible, almost independently of the age of the culture, for ages from 2 to 9 days.

Results and discussion. The dark survival of the two strains is shown in Fig. 1, carried to a survival probability of about 10^{-4}. (Each point on the curves represents at least 500 macrocolonies.) About half of the points on each of the curves represent runs in which the deviations could be explained by changes in output of the lamp; the diploid (SC6) survival, known from 3 consistent runs made earlier, was used as an indicator of the lamp's intensity in a statistically significant comparison between haploid and diploid strains. While, therefore, the measured survival of the haploid is not accurate, and therefore unsatisfactory, it is definitely not exponential in these experiments. This differs from the conclusion of Caldas and Constantin(6), who obtained not only an exponential survival for the haploid but a greater haploid survival than diploid for survival below about 17%. Their diploid curve was similar to the one shown here. Possibly their haploid strain had become contaminated with diploid, as mentioned above, although this could not result in an exponential curve; however, since their observed points are not published it is difficult to determine the source of the difference.

While the two dark survival curves are not exponential, they become straight at high dose, and asymptotically parallel. On the basis of the target theory, this suggests that part of the action of the radiation is on the genetic structure, with 2m targets inactivated in the diploid and m in the haploid; from the curves, m would be of the order 3. If this mechanism is accepted, and the multiple-hit theory ruled implausible, one must then suppose that there is, superimposed on the direct action, an indirect action which becomes less important as the dose becomes great, since the haploid curve is sigmoid. One must further suppose that this indirect action is not significant at any dose when X-rays are used since the X-ray survival curve is exponential (5,6). It would be interesting to verify these speculations by, for example, studying the relative temperature dependence of the low and high survival parts of the two curves, and by an additivity study of X- and U.V. radiations.

The photoactivation is as striking as in the case of other organisms. Here only the curves for maximum reactivation (about 30 min.) are shown (Fig. 1), although rough data for intermediate curves (5, 10, 15 min.) were also taken. If one applies the reduced-dose principle(7) to these data, then (with P_o = survival for zero light ("dark"), P_{oo} = survival for infinite light ("light"), $P_o\ (qx) = P_{oo}\ (x)$ with $q<1$, x = dose. That is, the light survival at any dose is obtained from the dark survival at that dose reduced by a constant fraction q.) These data satisfy the relation quite well. By the theory of Novick and Szilard(3) this relation can be obtained by assuming that the effect of the radiation is to produce a poison, of which the fraction q can be decomposed by the visible light, as in a monomolecular reaction. That is, q appears as a characteristic parameter of the poison, for which the organism has a characteristic

sensitivity. Therefore it is highly suggestive that the q values for the haploid and diploid curves are 0.43, 0.48 respectively, *i.e.*, very close to each other (always noting, however, that these numbers are subject to about 20% error) and also close to the value obtained for *E. coli* by Novick and Szilard. It is also interesting to inquire whether in reactivation the initial effect is direct (*e.g.* by the induced decay of a previously excited metastable state) or "indirect" (as in the poison theory). First, it can be shown, in a purely formal way, that once the dose-reduction principle is accepted, then the initial lethal action of the radiation can not be through purely target or direct effects.‖ If the reactivation is through a metastable state, with or without superimposed chemical action that takes no part in the reactivation, then it can be shown that $P(x,t) = P_o(x)\, f(t) + P_{oo}(x)\, [1-f(t)]$ for ultraviolet dose x, light dose t, and where 1-f is the probability of inducing a transition to a "live" state from a dormant state; for a single metastable, for example, $f = e^{-at}$. In any event, the survival at an intermediate value of visible light dose is a linear interpolation between the extreme curves of dark, and maximum light, survival. The theory of Novick and Szilard, on the other hand results in $P = P(L)$, $L = xg(t)$ where $g(O) = 1$, $g(oo) = q$. While neither the intermediate values taken here (not shown), nor the published data of Novick and Szilard, fit the metastable state picture, these measurements are consistent with the poison theory, as is the work of the last named authors.

Summary. The survival fractions of diploid and haploid strains of *S. cerevisiae* after ultraviolet irradiation (2537 A.U.) have been obtained, compared, and modified by photoreactivation following the initial irradiation. The haploid survival follows a sigmoid curve and is smaller than the diploid at the same dose. The dose reduction principle (which can be successfully applied to the data) yields a reduction factor that appears substantially the same for photoreactivation of both strains. The data appear consistent with the model proposed by Novick and Szilard.

The author would like to thank Professor R. E. Zirkle for making his laboratory available during this work.

‖ This can be shown by an examination of the class of functions that result from the target theory, (see Timoféeff-Ressovsky, N. W., and Zimmer, K. G., Das Trefferprinzip in der Biologie, *Biophysik* I, (S. Hirzel, Leipzig, 1947)) and noting that if one of these represents the dark survival, then the light survival function cannot belong to the same class, except in the special case where both light and dark survival are exponential.

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Action of Pituitary Pressor Hormones on Unanesthetized Rabbits.* (19348)

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As appears from recent reviews(1,2) it is commonly assumed that pituitary pressor hormones produce a fall in blood pressure when applied to unanesthetized animals. Therefore decerebrated or deeply anesthetized animals are used for the assay of pressor activity of extracts of the posterior pituitary lobe.

However, Landis, Montgomery and Sparkman(3) and Page and Ogden(4) obtained a pressor effect in unanesthetized rabbits. These authors used capsular methods for the estimation of blood pressures. This method(5) has been used with success in the estimation of renin(6-8). The close similarity between the curves obtained with anesthetized animals after injection of renin and of pituitary pressor hormone (vasopressin, pitressin) suggested a study of the action of this hormone in unanesthetized animals, because anesthetization alone produces an unphysiological fall in blood pressure(9,10).

Experiments and results. The pressor hormone used (Insipidin†) contained 20 international pressor units per ml. Dilutions were made in physiol. NaCl so that the amount administered to the rabbit was contained in 1-2 ml. This amount was injected intravenously in one ear and the blood pressure was measured on the other ear by means of Grant and Rothschild's capsule. Only one measurement was carried out on each rabbit each day in order to avoid possible secondary effects. The curves obtained were quite similar to those obtained by the usual measurements on the carotid artery of deeply anesthetized or decerebrated animals.

When the individual responses for the single doses are plotted in a log dose-response diagram Fig. 1 is obtained. It is seen that the mean values below a dose of 0.5 unit per kg

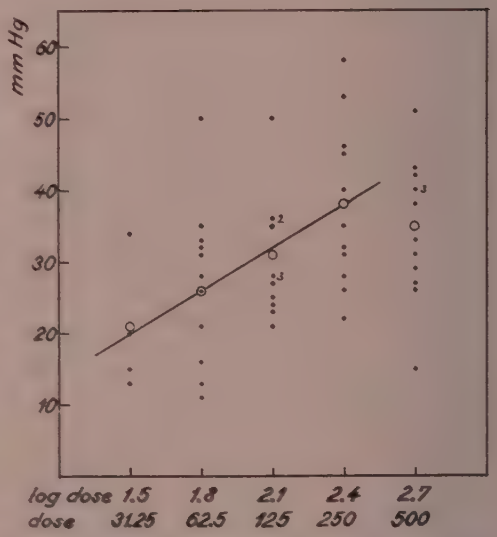

FIG. 1. Individual responses plotted with log dose (in milliunits/kg) as abscissa and blood pressure (mm Hg) as ordinate. The mean values are marked: O.

lie on a satisfactory straight line though the individual responses vary to a large extent. In the curve here presented the increase in response corresponding to a doubling of the dose is 6 mm Hg. When the measurements were repeated about 3 months later (partly with new animals) a similar curve was obtained, where a doubling of the dose represented an increase in blood pressure of 8 mm Hg. The same type of response curve was found valid for renin by Pickering and Prinzmetal(6). When the effect of the same dose was measured once each day during 6 consecutive days (6 animals) the variations in response for each animal were far smaller than the variations of the different animals to the same dose, as represented by the points in Fig. 1. A few animals behave so irregularly that they must be excluded from the measurements. The same was found true in the estimations of renin.

Discussion. Contrary to the views expressed by a number of previous investigators(1,2) the

* "Nordisk Insulin Fond"; Copenhagen, has supported these blood pressure investigations. The skillful assistance of Mrs. Kirsten Hviid is acknowledged.

† From M. Alfred Benzon, Copenhagen.

pituitary pressor hormone exerts a pressor action on unanesthetized rabbits. This effect is so constant that it may be used in a new and convenient method for the estimation of the potency of pressor hormone preparations. Too few animals were available to us for a thorough investigation of the accuracy of this method. In previous experiments with unanesthetized animals the fall in blood pressure is often followed by an increase(9). As the pituitary glands used by the older investigators were often air dried, enzymatic activity and bacterial growth during the drying process may have caused contamination with depressor substances. Thus a proteolysis followed by bacterial decarboxylation might yield histidine and histamine.

The results here obtained show that doses of 0.5 unit per kg (and in other series also those of 1.0 and 2.0 units per kg) fall considerably below the curve. This is indicative of a secondary depressor mechanism interfering when larger doses are given. The reaction here involved is probably the constriction of the coronary arteries observed by other investigators.

In some experiments the hormone solutions were heated, either in 0.25% acetic acid as in the standard procedure for estimating the pituitary pressor hormone or in physiological saline. No change in activity was noticed. The pressure effect was not caused by a nonspecific reaction because repeated injections of proteins (lyophilized human plasma, egg albumin, edestin; 2.5 mg per kg) did not produce any significant rise (less than 10 mm, usually about 5 mm). Even after such sensitization an injection 4 weeks later did not produce any change, except a small transient decrease during the first 1-2 minutes.

Summary. The increase in blood pressure produced by injection of pituitary pressor hormone (pitressin, vasopressin) in unanesthetized rabbits may be used in the assay of this hormone.

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Uptake of Radioactive Vitamin B_{12} by Various Microorganisms. (19349)

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Microorganisms differ in their requirements for vit. B_{12}. Some can thrive successfully without any exogenous source of this vitamin, whereas others require it for propagation: still others have a need for vit. B_{12} only during the initial phase of growth. It is, therefore, of interest to study the possible incorporation of vit. B_{12} from the medium into bacterial cells of species representative of these three types. To this end, several strains of microorganisms were allowed to grow in a basal synthetic medium prepared according to Skeggs and Wright(1), to which radioactive vit. B_{12} (2) tagged with Co^{60} was introduced. The presence of the radioactive cobalt in the molecule facilitates the identification and tracing

TABLE I. Determination of Radioactivity in Cells Grown in Synthetic Medium Containing Vit. B_{12} Tagged with Co^{60} (8 mμg equivalent to 60 cpm).

Organism	Hr at 37°C	Spectrophotometer reading*	Total No. of microorganisms ($\times 10^9$)	Radioactivity in total microorganisms (cpm)	Molecules vit. B_{12} per microorganism
L. leichmannii	40	380	130	23	8
L. arabinosus	18	550	184	<3	<<1
Esch. coli	40	120	20	8	25
S. lactis R	23	180	32	25	40
B. subtilis	18	350	140	36	14
P. mirabilis	40	110	16	19	60
B. mycoides	22	195	36	20	30
Esch. freundii	40	125	20	24	60
S. dobson	40	200	96	23	12
L. lactis Dorner	24	250	44	23	25

* Spectrophotometer readings were obtained with Klett-Summerson photo-electric colorimeter using filter #42.

of that part of the vitamin molecule.

Experimental procedure. A. *Microorganisms chosen for study.* *L. leichmannii* is dependent on an exogenous source of vit. B_{12} for multiplication; while proliferation of *L. arabinosus* is independent of such a source. These strains were therefore studied as typical of the extreme cases. Exogenous vit. B_{12} can stimulate the growth of *L. lactis* Dorner during the initial phase of growth and it, therefore, is representative of the third category. Others were included in order to ascertain whether their requirements for vit. B_{12} if any, had any relationship to their uptake. B. *Measurement of the uptake of* Co^{60}. Varying amounts (4-8 mμg) of radioactive vit. B_{12}* containing $Co^{60(r.a.)}$ were added to 40-100 ml portions of media. After autoclaving, the enriched media were seeded with the selected organisms. At the end of the respective incubation periods listed in Table I the luxuriant growth which usually occurred was measured in terms of turbidity readings using a Klett spectrophotometer. Estimations of the number of viable and total microorganisms were performed by plate and direct counting methods respectively. The organisms were collected by centrifugation, washed twice with 0.85% saline solution and transferred into Kjeldahl flasks for digestion with nitric-sulfuric acid mixture. After complete destruction of the organic matter, the digests were transferred into beakers and the excess acid removed by heating. For measurement of radioactivity the inorganic residue was then transferred quantitatively to a planchet. As an alternative method, washed cells were placed directly in planchets. This latter method yielded results of essentially equal accuracy and since it is simpler, is the one of choice. In order to check the recovery of added radioactivity, it was necessary to estimate the vitamin remaining in the cell-free supernatant fluids. However, these fluids contained such a large amount of salt as to interfere with measurements of radioactivity. It was, therefore, essential to liberate the cobalt bound to the B_{12} molecule by autoclaving for 1-2 hr at 15 pounds pressure after addition of 3 to 4 ml of concentrated sulfuric acid in the presence of 0.5 ml of 0.1 N $CoCl_2$ carrier solution and 1 ml of 5% Na_2SO_3 solution. After cooling, the solutions were adjusted to pH 9-10 with 10 N NaOH solution and 1 ml of a 50% NaHS solution was added. The contents were heated over a steam bath for 15 minutes. The precipitates were collected and dried with acetone, and transferred quantitatively to planchets. To insure more complete recovery of Co^{60}, the supernatant fluids from the CoS precipitates were acidified to about pH 5-6 with 6 N HCl and heated on the steam bath until coagulation occurred. The resultant precipitates after centrifuging were washed with distilled water. Each was superimposed upon the planchet containing the previously separated CoS from the same

* The solution of radioactive vit. B_{12} kindly supplied by Merck and Company has a specific activity of 40 microcuries per mg of solid vitamin. Its microbiological activity was found to be identical with that of crystalline vit. B_{12} on an equal weight basis.

sample. Radioactivity was measured with a Cyclotron Specialties Counter equipped with a thin (3.1 mg/sq cm) mica end window counting tube.

Results and discussion. The results of determinations of uptake of radioactivity by different organisms are shown in Table I. They demonstrate that *L. leichmannii* which required vitamin B_{12} for growth took up radioactivity equivalent to about 25 cpm or about 4 mμg of the tagged vitamin in 45 ml vit. B_{12} media. On the other hand, *L. arabinosus,* for which an exogenous source of vit. B_{12} is not essential, took up negligible radioactivity. In the experiments with either organism, the radioactivity in the cell-free supernatant fluids and washings was also estimated. Our data showed that the total radioactivity in these fluids and in the intact cells accounted for virtually all of the radioactivity originally introduced into the system (90% for *L. leichmannii* and 85% for *L. arabinosus*).

In addition to these two cases, the uptakes of radioactive vit. B_{12} by 8 other strains are included in Table I. They demonstrate that 8 microorganisms could incorporate vit. B_{12} in the cells, even though they could grow equally well in the absence of this vitamin. It is worth noting that *L. arabinosus* was the only organism in our list which failed to show appreciable radioactivity in the cells.

The data on the last column of Table I give the approximate number of molecules of radioactive vit. B_{12} taken up by various growing organisms. These numbers varied from 10 to 60 molecules per organism, with the exception of *L. arabinosus,* whose uptake was essentially nil. The results of a typical experiment which illustrate the method of calculation showed that when 8 mμg of radioactive vit. B_{12} were added to 50 ml media, 3-4 mμg were found in the cells after 45 hours of incubation. The number of living cells in the media was estimated on a very small aliquot sample to be 1.7×10^{11} by plate count. Assuming the molecular weight of vit. B_{12} to be 1400(3), it was readily calculated that approximately 8 molecules of the vitamin were incorporated in each *L. leichmannii* organism. This is necessarily a maximal ratio since presumably radioactivity is a measure of vit. B_{12} in both dead and viable organisms while plate counts gave an estimate of living organisms only.

In order to ascertain whether the uptake of radioactivity by an organism such as *L. leichmannii* is due to vit. B_{12} *per se,* or to the formation of some cobalt complexes, the following experiments were performed. In one, comparable amounts of radioactive cobaltous chloride were used instead of radioactive vit. B_{12} in uptake experiments similar to those described above. Except where the strain under investigation absolutely required an exogenous source of vit. B_{12} (*e.g., L. leichmannii*) this series was carried out both with and without inclusion of non-radioactive vit. B_{12} in the media in amounts of 4-8 mμg. The data, except with *L. leichmannii,* demonstrate that the strains studied grew equally well regardless of the inclusion of B_{12} in the media and that in every case, including *L. leichmannii,* there was a negligible radioactivity in the washed cells even when the quantity of labeled $CoCl_2$ added was a several-fold excess over that offered as radioactive vit. B_{12} in the first series. These results, therefore, demonstrate that simple Co compounds are not retained in the cells and, inferentially, that the Co atom was not readily released from the vit. B_{12} molecule since there was no interchange between stable Co of the vit. B_{12} molecule and Co^{60} from the $Co^{60}Cl_2$.

In another experiment designed to explore further whether Co^{60} is likely to be present in the protoplasm as a part of the vit. B_{12} molecule, we disintegrated the bacterial cells (such as *L. leichmannii*) with an all glass homogenizer according to the procedure of Dochstader and Halvorsan(4). The homogenates, containing super-cell hyflo and cellular debris,

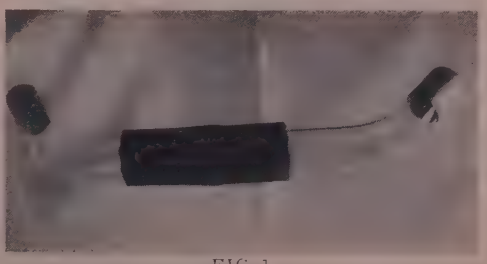

FIG. 1.

TABLE II. Determination of Radioactivity in *L. leichmannii* and a Second Microorganism Grown in a Medium Containing Radioactive Vit. B_{12}. (8 mμg equivalent to 100 c.p.m. added to each competitive system.)

	Total radioactivity in organisms (c.p.m.)	
	×*	*L. leichmannii*
L. arabinosus	3	34
Esch. coli	17	32
S. lactis R	28	26

* × = second organism.

were extracted with distilled water, and the aqueous extract analyzed for vit. B_{12} activity by the microbiological method of Skeggs and Wright(1). The results indicated that the vit. B_{12} measured microbiologically checked within 20% with that calculated from radioactivity due to Co^{60}.

Since the data obtained from the uptake experiments demonstrate that pure cultures of microorganisms incorporate radioactivity into their cells over a varied range when offered radioactive vit. B_{12}, it would be of interest to observe the fate of this tagged vitamin when introduced into a system of competitive organisms as is found in the intestinal tract. However, the physical separation of the (cultural) components of a mixed culture is, at present, a most difficult, if not impossible task. A partial solution of the problem of assaying individual species of bacteria for radioactivity while permitting all organisms access to media (containing Co^{60} labeled B_{12}) would be to grow such cultures in cellophane bags or in systems separated by cellophane membranes. In this way the organisms would be readily separable but the permeable barriers would allow change of low molecular components of the media such as vit. B_{12} as well as the metabolic products of the organisms under investigation. Fig. 1 is an illustration of the apparatus employed for this purpose. Table II summarizes the measurements of radioactive B_{12} uptake by organisms in the competitive systems, as estimated by the procedures outlined previously, and shows that *L. leichmannii* had taken up the same proportion of the added activity as when grown alone. Similar results of competitive systems of *L. leichmannii* against other bacterial organisms are also given in Table II.

Summary. A number of microorganisms were grown in a synthetic medium in which was incorporated radioactive vit. B_{12} tagged with Co^{60}. Among those studied, radioactivity was found in varying amounts in the cells of all species studied except *L. arabinosus*. The incorporation of the radioactivity was not related to the requirement of vit. B_{12} for growth of the bacterial cells. It was estimated that approximately 8 molecules of the tagged vitamin united with each *L. leichmannii* cell. Evidence is presented to show that the radioactivity in the cell is due to vit. B_{12} in that (1) cells grown in the presence of $Co^{60}Cl_2$ with or without addition of non-radioactive vit. B_{12} contained no radioactivity, and (2) the microbiological activity in homogenates of distintegrated cells paralleled that calculated from radioactivity due to Co^{60}. Measurements of uptakes of radioactive vit. B_{12} by two competing organisms (such as *L. leichmannii* and *E. coli*) separated by cellophane membranes readily permeable to this vitamin as well as other nutrients and metabolites demonstrate that radioactivity entered the cells of both organisms in essentially the same amounts as when each species was grown alone.

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Effect of Raw Soybean Meal on Growth of the Chick. (19350)

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We have shown(1,2) that in diets containing exclusively raw or cooked soybean meals as sources of protein, approximately 5% of raw soybean meal protein is associated with all or nearly all of the growth inhibition for the chick that may be observed with all-raw soybean protein in the diet. The manner in which the growth response changes with changing proportions of raw to cooked soybean meal in the diet may reveal much concerning the mechanism of the inhibiting effect.

Methods. The soybean meal diets were the same as used previously(1,2) except for the omission of the methionine. The remainder of the diets was composed of vitamins, minerals, and glucose. The diets were fed to New Hampshire chicks which were distributed into groups of 18 containing 9 of each sex. We shall for convenience refer to levels of soybean protein in discussing methods and results, although it is understood that the growth inhibitor is not necessarily an integral part of this protein.

Growth results. Results with the 20% protein diets are given in Table I, and plotted as the lower curve in Fig. 1. Practically all the growth inhibition was attained at the 5% raw protein level. There was only a slight further effect on growth at higher levels of raw protein. These data confirm our previous report(2).

Results with the 30% protein diet are given in Table II. In this case, also, approximately all of the growth inhibition was attained at the lower levels of raw protein. Since the protein increments were 5%, the point of inflection of the response curve is not as accurately established. All the chicks grew faster than the chicks at the 20% protein level.

TABLE I. Effect of Various Levels of Raw Soybean Meal on Growth of Chicks Fed a 20% Protein Diet.

Raw soybean meal protein in diet, %	Cooked soybean meal protein in diet, %	Avg wt of chicks at 14 days on diet, g
20	0	84
17.5	2.5	86
15	5	86
12.5	7.5	86
10	10	90
7.5	12.5	89
5	15	90
2.5	17.5	102
0	20	128

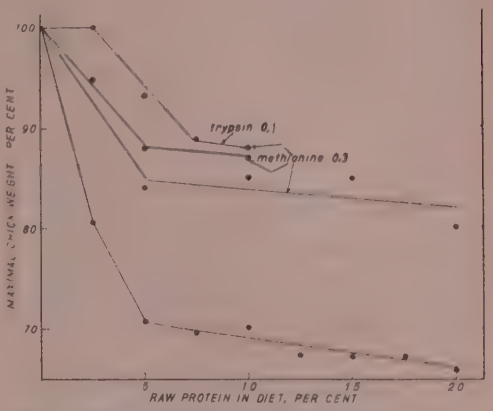

FIG. 1. Growth of chicks on mixtures of raw and cooked soybean meals which supply all the protein in a 20% protein diet. Supplements added as indicated.

TABLE II. Effect of Various Levels of Raw Soybean Meal on Growth of Chicks Fed a 30% Protein Diet.

Raw soybean meal protein in diet, %	Cooked soybean meal protein in diet, %	Avg wt of chicks at 16 days on diet, g
30	0	142
25	5	133
20	10	130
15	15	152
10	20	154
5	25	176
0	30	186

Discussion. If the principal growth inhibition associated with raw soybeans were caused by the reduced availability of some essential nutrient, such as methionine, the growth of chicks should drop progressively over the range from none to all-raw protein. Actually, the inhibition develops rather abruptly at first

and thereafter the growth decreases only slightly as the proportion of raw protein increases. A "toxicity" should become more acute as the threshold of tolerance for the toxic substance is approached, that is, the toxicity should be manifested more distinctly at the higher levels of raw meal. In such case the 30% protein diets would be even more toxic than the 20% protein diets. It is plain that these expectations are not fulfilled.

A mechanism in which a component of the proteolytic system in the chick is inactivated, while the remainder of the system is allowed to function at a reduced but fairly constant efficiency, is concordant with the fact that the growth inhibition is brought to approximately full effect when raw soybean meal supplies only one-fourth of the total protein. More specifically, such an effect would be expected from the antitrypsin of soybeans if this anti-enzyme had little or no influence on the proteolytic enzymes other than the trypsin secreted by the chick.

In Fig. 1, we have plotted the growth results from this study and our earlier studies(1,2) with 20% protein diets. In order to compare data obtained at various times and for slightly different experimental periods, the growth was expressed as a percentage of the maximal obtained in each experiment. Special supplements have been indicated on Fig. 1. The curves show that, as would be expected(4,5), there was a greater range of the inhibitor effect when the moderate methionine deficiency was not corrected (lower curve). The addition of trypsin (1-110) caused a displacement of the growth response curve to the right, after which the curve resumed the same pattern as its control curve. This indicates that the added trypsin preparation was able to neutralize a definite amount of the growth inhibitor.

The effect of trypsin added to raw soybean meal diets may be entirely masked if there is enough inhibitor in the diet to overcome the trypsin added plus that secreted by the animal. This fact is demonstrated further by our results with varying levels of trypsin (1-300) added to the 20% protein diet containing 5% raw protein. The data given in Table III show that 0.15% of the trypsin counteracted all of the growth inhibition while .05% trypsin had no effect. Recently it has been reported that a relatively enormous level of crude trypsin added to a 25% raw soybean meal diet for rats will counteract the growth inhibition(3).

TABLE III. Effect of Trypsin in Counteracting Growth Inhibition Caused by 5% Raw Soybean Protein in Chick Diets.*

Trypsin added to 5% raw protein diet,† %	Avg wt of duplicate chick groups at 14 days, g
.00	115
.05	111
.10	123
.15	161
.00‡	162

* Diets contained a total 20% soybean protein plus .3% methionine.
† Trypsin (1-300).
‡ All-cooked protein diet.

The nature of the flatter portions of the curves in Fig. 1 suggests a proportional growth inhibition of a type different from that involving trypsin. By projecting the flat portions to the left, an estimate may be obtained of the growth that would result if the trypsin effect only were in operation. Subtracting this from the total growth inhibition leaves an estimate of the second effect. This is found to be, without methionine added—6% and with methionine added—4 to 6%. Evidently, this second effect upon growth is relatively small and independent of added methionine. It may be most simply explained perhaps, as a result of a general decrease in availability of nutrients in raw as compared to properly cooked soybean meal.

Summary. Exclusively soybean protein diets, containing varying proportions of raw to cooked soybean meals were fed to young chicks. The growth inhibition associated with raw soybean meal was almost fully developed when one-fourth of the protein of either 20% or 30% protein diets was supplied in the raw form. The results do not fit the concepts of an unavailable essential nutrient or of a toxic component in raw soybean meal. The results are explainable on the basis of an antitrypsin which readily inactivates trypsin but permits the remaining proteolytic system in the digestive tract to function at a lowered degree of

efficiency. The further slight inhibition of growth with increased proportions of raw soybean meal is probably an effect of an entirely different nature and may be a manifestation of generally decreased availability of the nutrients in the raw meal.

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Distribution of Vitamin B_{12} in the Organs and Tissues of the Chick.* (19351)

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The vit. B_{12} content of various organs and tissues of the rat was first reported by Lewis, Register, and Elvehjem(1). These investigators used a rat growth assay to determine vit. B_{12} content. Several laboratories have published microbiological data on the vit. B_{12} content of some of the organs of the chick (2-4). Only a few organs were tested and no attempt was made to correct for desoxyribosides which interfere in the microbiological assay.

The purpose of this report is to present the results of studies of the vit. B_{12} content of the tissues and organs of the chick using a microbiological assay procedure. Corrections were made for desoxyribosides present in the samples. The rat method was not used because its low sensitivity makes it unsuitable for assaying those tissues which contain small quantities of vit. B_{12}.

Experimental. Thirty day-old Single Comb White Leghorn cockerels, obtained from a commercial hatchery, were used in the experiment. The chicks were housed in electrically heated battery brooders with wire-mesh floors. Food and water were supplied *ad libitum.* An alpha-protein-cerelose diet adequate in minerals and all known vitamins was used(5). The diet was supplemented with 50 μg of vit. B_{12} (Cobione) per 100 g. After 30 days on this regime the chicks were sacrificed and the carcasses frozen. The carcasses were removed from the freezer in groups of 5 and the various organs and tissues removed and weighed. The samples were pooled, homogenized in a Waring Blendor and then autoclaved for 30 minutes in acetate buffer at pH 4.5. After autoclaving, the samples were cooled, adjusted to a convenient volume, filtered and the filtrates assayed. This method of treatment was found to yield consistent results and no further increase in vit. B_{12} values was obtained by enzymatic digestion with pepsin, trypsin, or pancreatin. The assay medium of Peeler *et al.*(6) was employed using *Lactobacillus leichmannii* ATCC 4797. Duplicate assays were conducted on each sample. The alkali treatment used to correct for desoxyribosides was that of Hoffmann *et al.*(7).

Results and discussion. The vit. B_{12} content of the tissues and organs is presented in Table I. The desoxyriboside correction was negligible in most samples. The high level of dietary vit. B_{12} resulted in a very wide ratio between the vit. B_{12} and desoxyriboside content of the organs, thus the interference of

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TABLE I. Distribution of Vit. B_{12} in the Chick.

Sample	Vit. B_{12} content of moist sample, mμg/g	Range, mμg/g
Kidney	1107 (3)*	1060-1145
Liver	444 (3)	402- 480
Pancreas	425 (3)	334- 479
Proventriculus	281 (4)	231- 332
Testicle	244 (3)	235- 257
Heart	201 (4)	188- 229
Adrenal	178 (6)	137- 242
Spleen	155 (3)	128- 191
Gall bladder	138 (3)	94- 172
Thyroid	115 (3)	101- 134
Thymus	109 (3)	98- 115
Small intestine	82 (5)	69- 100
Lung	60 (6)	43- 75
Brain	56 (4)	42- 77
Gizzard	45 (6)	33- 67
Leg muscle	36 (6)	30- 40
Breast muscle	29 (3)	24- 34
Eyeball	8 (5)	7- 8.4

* Figures in parentheses represent number of groups assayed.

Organs and tissues of 5 chicks were pooled in each group.

desoxyribosides in the microbiological assay was minimized. Kidney was found to contain the highest concentration of vit. B_{12}. Liver and pancreas are next in potency, followed by the proventriculus and testicles. Muscle tissue, except for the heart, is relatively low in vit. B_{12}. The eyeball contains the lowest concentration.

Because of the high level of dietary vit. B_{12} used in this experiment, the vit. B_{12} content of the tissues and organs is probably higher than the levels found in chicks receiving normal amounts of this vitamin. This is evident from the report by Couch and Olcese (4). These investigators employed a corn-soybean meal diet supplemented with 2 μg of vit. B_{12} per 100 g. They obtained the following vit. B_{12} values per g: liver, 186 mμg; kidney, 83 mμg; pancreas, 73 mμg; and spleen, 124 mμg. The values reported in this paper are all higher than those obtained by Couch *et al.* This indicates that certain organs, particularly the kidney, liver, and pancreas, have the ability to store appreciable quantities of vit. B_{12}. The relative order of potency of the various organs and tissues of the chick observed in the work reported in this paper agrees fairly well with the results obtained by Lewis *et al.*(1) in the rat.

Summary. Microbiological data have been presented on the distribution of vit. B_{12} in the tissues and organs of chicks fed a purified diet containing a high level of vit. B_{12}. Kidney was found to contain the most vit. B_{12} while liver and pancreas contain lesser quantities. Muscle, except for the heart, is low in vit. B_{12} under these conditions. A comparison of these results with other published data indicates that certain organs, particularly the kidney, liver and pancreas, have the ability to store appreciable quantities of vit. B_{12}.

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Multiplication of the H37Rv Strain of *M. Tuberculosis* on Blood and and Its Derivatives.* (19352)

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In previous studies(1-3) it was demonstrated that *M. tuberculosis var. hominis* and recently isolated tubercle bacilli were capable of initiating growth from small inocula on a variety of basal media to which human blood had been added. These included Bordet-Gengou agar, blood agar base, nutrient agar, and agar-agar alone. The addition of serum, plasma, or fractions thereof to synthetic media have yielded conflicting results(4-6). Youmans and Youmans(4) observed that bovine proantithrombin (fraction III-1) when added to Prauskauer-Beck liquid medium increased the rate of multiplication of the H37Rv and freshly isolated strains to the same degree as did whole serum. Less stimulation was obtained with other fractions, among which were included two globulins, prothrombin (III-2) and fibrinolysin (III-3). In the experiments herein reported various constituents of blood have been examined for their ability to serve as a complete nutrient for the tubercle bacillus. The data indicate that the problem is complex and that growth promoting properties are present in both the plasma and cellular components of blood.

Method. The preparation of standard and blood media and the technic of wet weight inoculation are given in detail in previous publications(1,2). The agar-agar was washed three times with sterile distilled water prior to use. In Table I human bank blood collected in ACD solution 3 weeks previously was utilized. The specimens of blood in Table II were drawn with a dry syringe and needle. Half was permitted to clot and the remainder was defibrinated in a flask containing sterile glass beads. The defibrinated specimen was utilized as such. Aliquots of this material were removed, the red blood cells were washed 3 times with sterile saline, and then diluted to proper concentration based on volume of packed cells. The plasma fractions, except for the lipoproteins, were obtained in the dried state. Weighed samples were dissolved in sterile saline and put through a Seitz filter. Most of the fractions were readily soluble but a few contained slight residues which were lost on filtration. Post-filtration precipitates were observed in certain of the mixtures particularly I and II, and I and III. The same procedures were followed for the bovine plasma fractions, namely prothrombin, proantithrombin, and fibrinolysin. All solid media were prepared in a volume of 4 ml and the liquid media were tested in 2 ml quantities. The hemoglobin was obtained from Nutritional Biochemical Corporation. It was characterized as a crude bovine product containing 14.5% nitrogen and 0.17% iron. Since a considerable portion was insoluble, fractional sterilization was attempted without success. It was decided, therefore, to sterilize at 17 lb pressure for 6 minutes (123°C). The specimens of ox hemin and globin were obtained from the same source and were classed as highly purified. However, practically none of the material was soluble in physiological saline and it was sterilized either by heating to 70°C for 10 minutes on 3 successive days or else by autoclaving for 10 minutes at 17 lb pressure.

Experimental. In the initial experiment shown in Table I, it may be seen that both plasma and red blood cells in proper concentration are capable of initiating growth of the H37Rv strain from inocula consisting of as little as 0.000001 mg or between 5 and 50 organisms. The plasma appears to be somewhat less nutritive but superior to red blood cells, particularly if one considers the fact that 10% washed cells is roughly equivalent to the amount present in 20% whole blood. In other experiments serum was used either in comparison with or instead of plasma and proved just as efficacious. Data of this type were typical when whole banked blood or the constituents thereof were incorporated into agar-agar.

* Aided by a grant from the Truman Collins Fund.

TABLE I. Growth of *M. tuberculosis* on Human Bank Blood and Components Thereof Incorporated into Agar-Agar.

Component tested	%	Inoculum of H37Rv in mg						
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Bank blood	10	7	14	14	14	14	21	35
	5	7	9	14	14	16	16	35
	2.5	14	14	14	21	16	35	35
	1	14	14	28	28	28	28	—
	.5	28	28	28	28	35	35	—
Washed red blood cells	10	7	7	14	14	14	14	—
	5	7	7	14	14	16	16	—
	2.5	7	7	14	14	16	16	—
	1	14	42	—	—	—	—	—
	.5	—	—	—	—	—	—	—
Plasma	10	14	14	14	14	16	16	35
	5	14	14	14	14	16	16	—
	2.5	9	14	14	16	16	21	—
	1	14	21	21	28	—	42	—
	.5	35	35	42	42	42	—	—

Numbers indicate day on which grossly visible colonies were first noted.

Since it was entirely possible that the agar may have contributed in some manner to the ability of tubercle bacilli to grow on blood, it was decided to utilize defibrinated blood, serum and washed cells in a saline menstruum. In these experiments freshly drawn blood was employed and to avoid hemolysis the serum was removed as soon as clotting had occurred. In order to rule out a possible stimulating effect of anticoagulants, a portion of the whole blood was defibrinated and either used as such or the red cells were removed and washed thoroughly with saline. Some of the data are presented in Table II and represent single specimens drawn from 6 normal adults of a total of 10 tested. It is apparent that both washed human red cells and serum are capable, under proper conditions, of supporting growth of inocula ranging from 0.001, 0.0001, and 0.00001 mg of tubercle bacilli. In the experiment presented, this represented starting inocula of approximately 50,000, 5,000, and 500 organisms, respectively. Growth of the H37Rv strain was approximately equal in both the defibrinated blood and washed red blood cells, the endpoint varying between 0.5 to 2.5%. It should be pointed out that the final readings represent smears made with a single drop from a 1 ml pipette. If loops were used to prepare the smears, the data were entirely irregular because of the fact that the organisms tended to grow in such tight skeins that they were not picked up.

The specimens containing serum were examined both macroscopically and microscopically at intervals and the final readings represent the degree of growth after 8 weeks. The results were unexpected and as can be seen from Table II, 2 major patterns of growth in serum were observed. The first type illustrated by J, B, and W, were specimens in which organisms grew in the greater but not in smaller amounts of serum. The least concentrations showing growth varied from 1.0 to 2.5%. The second type illustrated by Ja, G, and D was characterized by a zone in which concentrated serum proved inhibitory and with progressive dilution, an amount which supported growth was finally reached. This varied for each of the serums tested and in general, the final degree of growth was less than in the first type.

Attempts were made to identify more accurately the components in plasma capable of initiating growth of small inocula of tubercle bacilli. For this purpose various fractions were obtained and tested either in saline or incorporated into agar-agar.† In experiments of this type using physiological saline as a menstruum growth was demonstrated with combinations as follows: I,V; I,V,IV;

† The plasma fractions were supplied through the generosity of Dr. L. D. Wojick of Harvard University Laboratory of Physical Chemistry; Dr. H. D. Anderson of the Michigan Department of Health Laboratories; Dr. F. D. Johnson of the Cutter Laboratories.

TABLE II. Growth of *M. tuberculosis* on Human Blood and Components Thereof.

Sample	%	Inoculum of H37Rv in mg: Defibrinated blood 10^{-3}	10^{-4}	10^{-5}	Red blood cells 10^{-3}	10^{-4}	10^{-5}	Serum 10^{-3}	10^{-4}	10^{-5}
J	10	+	+	+	+	+	+	+++*	++	+
	5	+	+	+	+	+	+	+++	+	+
	2.5	+	+	+	+	+	+	++	+	—
	1	+	+	+	+	+	+	+++	++	++
	.5	+	+	—	+	—	—	—	—	—
	.1	—	—	—	+	—	—	—	—	—
B	10	+	+	+	+	+	+	+++	+++	+++
	5	+	+	+	+	+	+	++	++	++
	2.5	+	+	+	+	+	+	+	+	—
	1	+	+	—	+	+	—	—	—	—
	.5	—	—	—	—	—	—	—	—	—
	.1	—	—	—	—	—	—	—	—	—
W	10	+	+	+	+	+	+	+	+	+
	5	+	+	+	+	+	+	+++	+++	++
	2.5	+	+	+	+	+	+	+++	++	++
	1	+	+	—	+	+	+	+++	+	+
	.5	+	—	—	+	+	+	—	—	—
	.1	—	—	—	—	—	—	—	—	—
Ja	10	+	+	+	+	+	+	—	—	—
	5	+	+	—	+	+	+	—	—	—
	2.5	+	+	+	+	+	+	—	—	—
	1	+	+	+	+	+	+	+	—	—
	.5	+	+	+	+	+	+	+	+	—
	.1	+	—	—	+	—	—	—	—	—
G	10	+	+	+	+	+	+	++	—	—
	5	+	+	+	+	+	+	—	—	+++
	2.5	+	+	+	+	+	+	++	++	++
	1	+	+	+	+	+	+	+	—	—
	.5	+	+	+	+	+	+	—	—	—
	.1	—	—	—	—	—	—	—	—	—
D	10	+	+	+	+	+	+	—	—	—
	5	+	+	+	+	+	+	+	—	—
	2.5	+	+	+	+	+	+	—	—	—
	1	+	+	+	+	—	—	—	+	—
	.5	+	+	+	+	—	—	++	+	+
	.1	—	—	—	—	—	—	—	—	—

Growth determined at 8 wk from stained smears.
* Degree of growth measured in serum only since colonies were visible.

I,V,II,III; I,V,III,IV.‡ In evaluating these data it should be borne in mind that the inocula which grew were relatively heavy and represented between 5 million and 500,000 organisms. In a number of additional experiments in which the concentration of fractions I, II, II-1,2, III-0, III-1, III-2, III-2,3, IV-1, IV-4, V, VI, and lipoproteins was varied, multiplication of tubercle bacilli never occurred with any single one when an inoculum of 50,000 or less was used. We have at times, obtained growth with certain combinations of fractions incorporated into agar-agar, but the results were not considered reliable because of the stimulating influence of the solidifying agent. In addition to the plasma fractions from human blood it was possible for us to study relatively purified components of bovine plasma in the same manner.§ Also included are samples of bovine hemoglobin, hemin, and globin. All of these were examined in a saline menstruum and growth was estimated macroscopically where possible, and finally by the smear method. It may be noted that none of the bovine plasma fractions was capable of growing an inoculum of 0.0001 mg or less of the H37Rv strain but that colony formation was evident with larger numbers of organisms. The data do not indi-

‡ Fraction V 2.5 mg/ml; all others 0.5 mg/ml.

§ Obtained through the generosity of Dr. E. C. Loomis of Parke-Davis and Co.

TABLE III. Growth of *M. tuberculosis* on Bovine Blood Derivatives.

Component	mg/ml saline	Inoculum of H37Rv in mg					
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Proantithrombin	20	20	30	—	—	—	—
	10	30	—	—	—	—	—
Fibrinolysin	20	20	—	—	—	—	—
	10	30	—	—	—	—	—
Prothrombin	20	20	50	—	—	—	—
	10	20	50	—	—	—	—
Hemoglobin	15	6	12	20	30	30	50
	10	6	12	20	30	50	—
	5	6	12	20	50	50	50
	1	6	12	—	—	—	—
Globin	28.6	—	—	—	—	—	—
Hemin	12.8	—	—	—	—	—	—
Globin + hemin	—	—	—	—			
Lowenstein	—	6	10	12	12	16	—
Petragnani	—	8	10	12	14	16	—
10% human blood + agar	—	6	6	10	12	12	17

Numbers indicate day after inoculation on which growth was first observed.

cate that one fraction was more efficacious than any other (Table III).

The result with bovine hemoglobin was not surprising since human hemoglobin solutions obtained by lysing and sedimenting the stroma of human red blood cells were also capable of initiating growth from small inocula. The data show that the limiting concentration of the hemoglobin lies somewhere between 1 and 5 mg and that an inoculum of between 5 and 50 organisms will suffice. In some tubes growth was evident in 6 days. The tubercle bacilli on microscopic examination showed marked skeining and were typical in appearance. Similar results have also been obtained with bovine hemoglobin incorporated into agar-agar. On the other hand bovine hemin, globin, or combinations thereof in a variety of concentrations have never shown evidence of supporting growth of the H37Rv strain. The data regarding these derivatives of hemoglobin are by no means final since the products studied were purified but denatured.

Discussion. In the studies presented it has been observed that human blood contains all of the ingredients necessary to grow an inoculum of between 5 and 50 tubercle bacilli. For this purpose a minimal concentration of 1% defibrinated blood in saline is required. Multiplication is relatively rapid and in more concentrated specimens of blood, occurs within 2 to 3 weeks after inoculation. Variations in the ability of individual bloods to grow tubercle bacilli were observed, but were not considered to be significant. Further inquiry has revealed that both red blood cells, plasma, and serum are capable of supporting multiplication of the H37Rv strain. With regard to the red blood cell, evidence is presented that the active ingredient is hemoglobin. Although the material used was of bovine origin and relatively impure, it seems unlikely that any other constituent could be responsible if one considers that as little as 5 mg per ml was sufficient to grow a minimal inoculum of between 5 and 50 organisms. The fact that hemoglobin acts as a complete nutrient coincides with numerous observations on the essentially aerobic respiration of *M. tuberculosis*(7). It may also be recalled that cytochromes and prophyrins(8,9) have been identified as constituents of tubercle bacilli and other acid fast organisms. The factor in hemoglobin which is responsible for growth appears to resist denaturation and precipitation by autoclaving. Its relationship to the X and V substances of *Hemophilis* are under investigation.

Plasma and its constituents as growth promoting substances for the tubercle bacillus have usually been utilized as stimulants in an otherwise complete medium(4-6). In these studies it has been shown that multiplication occurs when plasma or serum is employed as a

sole source of nourishment. The fact that zones were demonstrated in certain of the specimens suggests the presence of inhibitors which are eliminated by dilution. It is also possible that the effect described may be due to concentration, physical state of the proteins, or to a change in oxidation-reduction potential under the conditions of the experiment. The data which have been obtained with the six major Cohn fractions of plasma cannot be considered as conclusive. They merely indicate that the factors necessary for the cultivation of tubercle bacilli in plasma fractions have not, as yet, been elucidated. Just why fractions I and V proved most efficacious requires further study.

The incorporation of bovine proantithrombin, fibrinolysin, and prothrombin either into agar-agar or into physiological saline did not result in growth of tubercle bacilli equal to that noted in human plasma or serum. Multiplication with large inocula occurred to the same extent in all three fractions and although our data are not comparable with those of Youmans and Youmans(4) they indicate that proantithrombin cannot, as yet, be assigned a special role as a plasma growth factor for the tubercle bacillus. In fact, the entire problem requires reinvestigation with appropriate technics. Thus even agar-agar as a solidifying agent in conjunction with blood or its products was responsible for contradictory results. It is believed, therefore, that any measurement of stimulation or inhibition due to the addition of a substance to a medium which by itself will support growth *M. tuberculosis* should be interpreted with caution. It seems even less likely that such data could be applied directly to infections in animals and man. Finally the demonstration that whole human blood and derivatives thereof are in themselves capable of furnishing a suitable environment for the multiplication of small numbers of tubercle bacilli makes it necessary to consider these substances and particularly hemoglobin, as factors in the pathogenesis of the naturally occurring disease.

Summary. It has been found that human blood, washed red cells, plasma, and serum contain all of the factors necessary for growing the H37Rv strain of *M. tuberculosis* from small inocula. The active component in red blood cells has been tentatively identified as hemoglobin. The essential ingredients in plasma or serum are more complex and require further study. A mixture of human fractions I and V was shown to be capable of initiating growth from large inocula but the data were not comparable with those obtained using whole serum. Similar results were obtained with bovine proantithrombin, fibrinolysin, and prothrombin.

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Observations on the Pharmacology of Mephenesin Carbamate.* (19353)

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With increasing clinical experience it has become apparent that, although mephenesin may represent an important therapeutic agent in the management of spasticity and hyperkinesis, the practical usefulness of the drug is limited by its short duration of action. In order to maintain a level of beneficial effectiveness, large doses must be given at frequent intervals. The short duration of mephenesin appears to be related to the loss of activity which follows the oxidation of the terminal hydroxyl group to form β-toloxy lactic acid (1). Berger and Riley(2) studied the acid succinate of mephenesin which, after intraperitoneal administration, appeared to have a longer duration of action than mephenesin. We have confirmed this observation but we have also noted that this difference in action is not present after oral administration.

Several active compounds,† in which the terminal hydroxyl group was blocked, have been studied. Among these, 1-methoxy-2-hydroxy-3-toloxy propane showed no advantage and the 1,2-methoxy-3-toloxy propane only slight prolongation of action. The carbamate of mephenesin, 3-o-toloxy, 2-hydroxypropyl carbamate (MC 2303), however, appears to offer some promise since, possessing comparable potency, it shows in addition a significantly longer duration of action than mephenesin.

In studies on white mice weighing 16-22 g, the carbamate was found to have a mean paralyzing dose on oral administration of 3.4 mM/kg as compared to 4.17 mM/kg for mephenesin. The lethal doses were 7.67 mM/kg for MC 2303 and 10.53 mM/kg for mephenesin (Table I).

Confirmatory studies of duration of action were done, using the effectiveness of the compounds against the extensor phase of maximal electric shock pattern as a criterion. Electroshock was induced by 60 cycle square wave current of 0.5 sec. duration and 8 milliamp strength. Table II shows the results of this type of study. It is evident that MC 2303 shows longer protection and greater potency.

Pilot studies were done to ascertain whether, in animals, the mode and site of action of the compounds could be considered comparable. In mice given MC 2303 the pinna reflex disappeared before the corneal, a sign which Toman(3) has considered characteristic of mephenesin-like activity. Anticonvulsant action in mice appeared similar, using both a modification of the intravenous titration technic of Orloff *et al.*(4) and the intraperitoneal injection of twice the median lethal dose of strychnine and of metrazol. In spinal and in anesthetized cats both compounds showed a selective depression of the multisynaptic flexor reflex. Similarities were also seen in the EEG changes of intact curarized cats and mesencephalic sectioned cats. Finally, in rabbits, doses of both drugs causing a smooth reversible paralysis (150-200 mg/kg IP) caused comparable slowing in the brain wave frequency with an increase in amplitude. In studies on the depolarization of isolated frog muscle, J. Wright(5) has noted comparable effects with mephenesin and MC 2303. Although mephenesin carbamate was somewhat more potent on a molar basis, it seemed possible that its action could be related to a degradation to mephenesin. This conjecture was disproven by extracting the 24-hour urine collection of 2 dogs given either mephenesin, or mephenesin carbamate orally using a modification of the method of Riley(6). Following mephenesin, β-toloxy lactic acid was recovered in 30% yield. The appearance of this compound in the urine could not be demonstrated after the carbamate was administered. The action of MC 2303 probably is not the result of its degradation to mephenesin.

Conclusion. MC 2303 is a compound simi-

* Aided by grant from E. R. Squibb and Sons.

† Submitted by Mr. W. A. Lott of E. R. Squibb and Son.

TABLE I. Mean Paralyzing and Toxic Doses of Mephenesin and MC 2303 in mM/kg.

	Intraperitoneal		Oral	
	ED_{50}	LD_{50}	ED_{50}	LD_{50}
Mephenesin	1.08 ± 8.8%	2.83 ± 3.8%	4.17 ± 5.1%	10.53 ± 5.1%
MC 2303	1.16 ± 7.9%	2.77 ± 5.9%	3.4 ± 5 %	7.67 ± 5.4%

TABLE II. Number of Animals Protected Against Extensor Phase of Maximal Electroshock Pattern. 10 animals per group.

Drug	Dose, mM	Time in min 10	40	70	120	180
Mephenesin	3.40	10	4	1	0	—
	2.30	8	1	1	0	—
	1.54	4	1	1	0	—
	.99	6	0	—	—	—
	.66	3	0	—	—	—
MC 2303	4.20	10	10	10	5	4
	2.76	10	10	6	0	—
	1.88	10	10	6	1	2
	1.25	10	7	1	0	—
	.81	8	2	0	—	—
	.54	3	0	—	—	—

lar to mephenesin in potency and activity, and appears to have a longer duration of action.

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Thiosemicarbazide Toxicity in Mice.* (19354)

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Domagk(1) has reported that the 3-thiosemicarbazones of substituted benzaldehydes are active *in vitro* in inhibiting the growth of *Mycobacterium tuberculosis*. Promising results have been reported for a number of these compounds when tested *in vivo* in mice(2-4). However, thiosemicarbazide (TSC) itself, although active *in vitro* in low concentrations against *M. tuberculosis*, var. *bovis*, strain BCG(5) has not proven effective when tested *in vivo* because of its extreme toxicity. Indeed, Dieke(6) has suggested using TSC as a rodenticide. She found that dose levels of 10-30 mg/kg caused convulsions and death within one to 3 hours in rats, dogs, cats, guinea pigs, and monkeys.

It has been shown(7) that the ciliated protozoan, *Tetrahymena geleii*, is inhibited by TSC and that the inhibition may be reversed at the lower dose levels by small amounts of pyridoxamine. Tests to determine whether or not the toxicity of TSC to mice can also be reversed by pyridoxamine were therefore carried out.

Methods. The animals employed in these experiments were in most cases 18-20 g C57 black mice. A few experiments were performed on Swiss and A/C3H hybrid mice, the results of which were in complete accord with those using the C57 black strain. The compounds tested were administered in water solution with the concentration adjusted so that a total dose was contained in one ml. Accurate oral doses were administered by a syringe with an attached blunted, curved 15-gauge hypodermic needle, which was introduced into the esophagus. Following administration of the desired compounds the animals were observed closely for 4 hours and again at 12- and 24-hour intervals. The time of death of each animal was recorded. The great majority of deaths occurred within the first 4 hours. It was observed that striking uni-

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TABLE I. Reversal of Thiosemicarbazide Toxicity. No. deaths/No. mice treated.

mg thiosemicarbazide/kg body wt	mg pyridoxamine/kg body wt								
	0	6.5	16	32	65	190	320	800	1600
12	2/5	1/5	0/5	—	—	—	—	—	—
65	5/5	—	—	5/5	2/5	0/5	0/5	—	—
160	5/5	—	—	—	—	—	—	1/5	—
320	5/5	—	—	—	—	—	—	—	2/5

formity occurred in the time of onset of convulsions in animals receiving the same dose of TSC alone. Often the first seizure was fatal. If not, successive seizures occurred at intervals of about 10 minutes until death ensued. All experiments were repeated many times. In all 810 mice were used.

Results. Doses of 20 mg of TSC/kg resulted in fatal convulsions in all animals tested. This dosage is in good agreement with the "maximal acceptable drug intake daily" for mice (16 mg/kg) as reported by Hamre *et al.*(4) and with the toxic range (10 to 30 mg/kg) found by Dieke(6) for 6 species of mammals other than mice. The importance of the presence of sulfur in the molecule in determining its specificity is demonstrated by the results with semicarbazide and aminoguanidine. If the sulfur is replaced by oxygen as in semicarbazide, the toxicity is reduced to about one-twentieth of that of TSC, whereas if an imino group is substituted as in aminoguanidine, no acute toxicity is encountered even with a dose of 650 mg/kg.

Table I gives data showing that complete reversal of the acute toxicity of TSC may be achieved by intraperitoneal injections of pyridoxamine (B_6NH_2). These data are representative of a large number of experiments. Uniform reversal of high doses of TSC (8 and 16 fold greater than the minimal toxic dose) was not obtained with the amounts of B_6NH_2 tested. However, when deaths did occur, they were delayed significantly beyond the time of death observed with TSC alone. Toxic levels of semicarbazide were reversed only sporadically with B_6NH_2. Neither pyridoxine nor pyridoxal was as effective as pyridoxamine in reversing the toxicity of TSC. In mice given daily intraperitoneal injections of 65 mg of TSC/kg, preceded by 320 mg of B_6NH_2/kg; for 9 days, no deaths, weight loss or other signs of toxicity occurred.

The possibility that the reversal of TSC toxicity by B_6NH_2 was due to a chemical interaction in the peritoneal cavity was ruled out by the fact that intraperitoneally injected TSC could be reversed by subcutaneous injections of B_6NH_2. The toxicity of TSC administered either orally or intraperitoneally was reversed by B_6NH_2 administered by either route. These data suggest that the site of reversal is in the tissues or the blood stream. It was found that B_6NH_2 was effective in overcoming TSC toxicity if injected within 3 hours before, or 30 minutes after TSC administration. If the B_6NH_2 was given 5 hours before the TSC, the initial convulsions and deaths were delayed significantly. Some protection was afforded when B_6NH_2 was given at the time at which the first death occurred in the control group (45 minutes after the TSC injection).

It was thought possible that the toxic effect of TSC might be enhanced if the B_6 content of the tissues was lowered by dietary restriction. Therefore groups of 40 mice were fed a diet deficient in B_6 (vit. B complex test diet, Nutritional Biochemical Corp.). Control groups of 10 mice were fed the same diet plus adequate amounts of B_6NH_2. After 27 days on these diets, the deficient animals had lost about one g/mouse, while the control animals had gained about 2 g/mouse. The toxic dose of TSC was then determined in these two groups of animals and compared to the toxic levels in animals fed the routine diet of Purina Chow. No significant difference in the TSC toxicity was observed.

The possibility existed that TSC toxicity might be due to the active removal of essential metabolites containing a carbonyl group, by thiosemicarbazone formation, and that these metabolites might be protected by administration of competing carbonyl compounds. Accordingly the following materials

were tested by intraperitoneal injection for their ability to reverse TSC toxicity or to potentiate the effectiveness of suboptimal doses of B_6NH_2: Na pyruvate (200 mg/kg), α-ketoglutaric acid (400 mg/kg), and glucose. These compounds had no effect. The administration of glutamic acid (400 mg/kg) and γ-amino butyric acid (up to 1625 mg/kg)(8) were also without effect. The convulsant effect of TSC does not appear to be due either to hypoglycemia or to hypocalcemia since glucose (650 mg/kg) and $CaCl_2$ (65 mg/kg) were inactive.

Discussion. It is not possible with the evidence at hand to come to a definite conclusion as to the mode of action of pyridoxamine in reversing TSC toxicity, nor is it clear why TSC is toxic. However, it would appear unlikely that pyridoxamine acts through its normal role of coenzyme for reactions involving transaminations, deaminations or amino acid decarboxylations. If this had been the case, one would have expected that TSC toxicity would have been greatly enhanced by B_6 deficiency. This was not found to occur. Also, the very large amount of B_6NH_2 required for reversal as compared to the amount needed for normal metabolism suggests that some sort of direct chemical inactivation takes place. In accord with this suggestion is the observation that an approximately equimolar amount of B_6NH_2 is required to neutralize a given dose of TSC. It also appears that the reversal of TSC toxicity by B_6NH_2 in mice takes place by a somewhat different mechanism than is the case with *Tetrahymena*(7). In *Tetrahymena,* protection is afforded by catalytic rather than stoichiometric amounts of B_6NH_2. Since keto acids are capable of detoxification of TSC in *Tetrahymena* cultures this may be correlated with the known function of B_6NH_2. In the mice, however, administration of large amounts of keto acids (pyruvic and α-ketoglutaric acids) produced no detectable alteration in the toxicity of TSC.

In *Tetrahymena,* TSC and semicarbazide are approximately equal in their inhibitory powers, and at the lower concentrations both are reversed by B_6NH_2. This is in contrast to the findings in the mice, where semicarbazide was only 1/20 as active as TSC, and was poorly reversed by B_6NH_2.

It would be of interest to extend the observations presented in this report to *in vivo* and *in vitro* studies of *M. tuberculosis.* Valuable information may be gained concerning the mechanism of action of TSC and of various thiosemicarbazones now being considered as agents in the chemotherapy of tuberculosis. It is also possible that B_6NH_2 may be found useful as an antidote for untoward reactions which may occur during clinical use of these compounds, if it is found that the inhibition of *M. tuberculosis* by TSC is not reversed by B_6NH_2.

Summary. 1. Fatal convulsions follow the administration of doses of 20 mg of thiosemicarbazide (TSC)/kg or more to mice. 2. Semicarbazide is approximately 1/20 as toxic as thiosemicarbazide, while aminoguanidine produces no toxic symptoms at a dose of 650 mg/kg. 3. The toxicity of thiosemicarbazide is completely reversed by stoichiometric doses of pyridoxamine administered either orally, intraperitoneally or subcutaneously. The administration of pyridoxamine is effective either preceding or following the dosage with TSC at appropriate intervals. 4. Dietary B_6NH_2 deficiency does not influence the toxicity of TSC. 5. Pyruvic acid, α-ketoglutaric acid, γ-aminobutyric acid, glutamic acid, glucose and calcium chloride did not reverse the toxicity of TSC nor enhance the activity of suboptimal doses of pyridoxamine.

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Endocrine Factors Affecting Weight and Ascorbic Acid Content of Rat Preputial Glands.* (19355)

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The preputial glands of the rat have generally been considered as accessory sexual organs whose size and function were dependent upon androgenic stimulation(1). Noble and Collip(2), however, suggested a direct effect of pituitary extract on preputial glands in addition to an indirect stimulation by corticotrophic and gonadotrophic principles. Jacot and Selye(3) reported that commercial preparations of adrenocorticotrophic hormone (ACTH) induced enlargement of the preputial glands of adrenalectomized-ovariectomized rats, which represented either an extra-adrenal activity of ACTH or a contaminating pituitary principle present in ACTH. Histochemical studies of preputial glands indicate the presence of ascorbic acid and sudanophilic lipids in the acinar cells, and steroids in the sebaceous secretory product(4). Because of these similarities to the adrenal glands, the present studies were undertaken to determine the normal ascorbic acid content of the preputial glands and to study various factors which affect their weight and vitamin content.

Methods. Exp. I. Thirty-four male rats of the Holtzman strain were adrenalectomized at the age of 33 days, and maintained for 3 weeks on 1% NaCl in the drinking water. Nine of these received .15 mg desoxycorticosterone acetate (DCA) daily, subcutaneously. Ten intact rats served as controls. Exp. II. Eighteen male rats were thyroidectomized at the age of 33 days and 11 intact rats were made hyperthyroid by addition of thyroid powder (0.5% Armour thyroid powder, by weight) to their diet. Twenty untreated rats of the same age served as controls. After 6 weeks, half of each group were stressed by immersing the animal (except the head) in water at 70°C for 5 seconds while under nembutal anesthesia, and autopsied one hour later. The other rats of each group were sacrificed without stress. The weights of the adrenal and preputial glands were recorded, and ascorbic acid determinations were made on each, using the method of Roe and Kuether (5). Exp. III. Thirteen male rats weighing 120-160 g, 6-7 weeks of age, were hypophysectomized. Twenty-four hours later the left adrenal and left preputial glands were removed, ACTH was injected by tail vein, and one hour later the right adrenal and right preputial glands were removed. The glands were cleaned, weighed on a torsion balance, and ascorbic acid content was determined.

Results. Exp. I. Single preputial glands of adrenalectomized rats, maintained on 1% NaCl in the drinking water, weighed 27.7 mg/100 g body weight, in contrast to a weight of 20.5 mg/100 g body weight for those of intact rats of the same age and sex (Table I). Daily injection of a maintenance dose of DCA to adrenalectomized rats resulted in preputial gland weight of 23.3 mg/100 g body weight.

Exp. II. Hypothyroidism for a period of six weeks following thyroidectomy resulted in a slight increase in preputial gland weight which was not statistically significant. Hyperthyroidism induced a more marked hypertrophy (Table I). Stress produced no statistically significant change in preputial gland weight. Ascorbic acid content of the control preputial glands was approximately 60 mg %, with no significant difference in those rats which were hypothyroid (65 mg %), or hyperthyroid (55 mg %). However, as can be seen in Table I, severe stress decreased the preputial gland ascorbic acid content of the controls by 27%; as compared with 89% in hypothyroid rats and 44% in hyperthyroid rats.

Exp. III. Rats hypophysectomized 24 hours previously showed an ascorbic acid content of the left preputial gland which was 78% that of controls (Table II). One hour after intravenous injection of various doses of ACTH (Armour LA-1A) to such rats, analysis

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TABLE I. Effect of Adrenal and Thyroid Function and Stress on Weight and Ascorbic Acid Content of Preputial Glands.

Treatment	No. of rats	Left preputial wt ± S.E. (mg/100 g body wt)	Ascorbic acid ± S.E. (mg/100 g tissue)
Control (8 wk old)	10	20.5 ± 1.6	—
Adrenalectomized (3 wk)	25	27.7 ± 1.5*	—
Adrenalectomized (3 wk) + .15 mg DCA/day	9	23.3 ± 2.2	—
Control (11 wk old)	10	18 ± 1.7	60 ± 2.8
Control, stressed†	10	17.1 ± 2.3	44 ± 7.7
Thyroidectomized	9	23.9 ± 2.2	65 ± 3.9
Thyroidectomized + stress†	9	15.3 ± 2.2	7 ± 3.8‡
Thyroid-fed	5	29.8 ± 3.2*	55 ± 3
Thyroid-fed + stress†	6	25 ± 4.1	31 ± 7 ‡

* Difference from controls statistically significant.
† Immersed in water at 70°C for 5 sec.
‡ Difference from non-stressed groups statistically significant.

TABLE II. Effect of Intravenous ACTH on Weight and Ascorbic Acid Content of Preputial Glands of Hypophysectomized Rats.

Treatment	No. of rats	Preputial wt ± S.E. (mg/100 g body wt)	Ascorbic acid ± S.E. (mg/100 g tissue)
Hypophysectomized (24 hr)	13	20.8 ± 1.3	47 ± 3
" + .25 μg ACTH	1	18.9	50
" + 1	3	26.8 ± 2.9	51 ± 8.1
" + 2	3	19.7 ± 1.2	56 ± 7.7
" + 4	3	21 ± 3.8	32 ± 5.8

of ascorbic acid content of the right preputial was made. The only appreciable effect was noted after the highest dose (4 μg/100 g body weight), which produced a 32% decrease from that of the uninjected hypophysectomized rats.

Discussion. Gemzell *et al.*(6) have shown that adrenalectomy in the rat is followed by more than a 30-fold increase in circulating ACTH. The results in the present adrenalectomy experiment indicate that preputial enlargement is produced by endogenous ACTH hypersecretion comparable to that observed by Jacot and Selye(3) following treatment of rats with commercial ACTH preparations. This makes it unnecessary to assume the presence of a preputial-stimulating contaminant in such preparations, although it does not preclude the possibility. Administration of DCA to adrenalectomized rats impaired the preputial hypertrophy which suggests that this steroid may have partially inhibited the ACTH hypersecretion. Increased weight of the preputial glands in hyperthyroid rats may be explained either on the basis of increased ACTH production(7) or increased androgen secretion from hyperactive interstitial cells of the testes(8). The statistically insignificant reduction of preputial weight in certain groups one hour after stress may be partly due to extrusion of pre-formed sebaceous secretion in response to sudden sensory stimulation. Actual cellular degeneration seems unlikely.

Ascorbic acid depletion in preputial glands following stress in normal, hypo- and hyperthyroid rats parallels the depletion of adrenal ascorbic acid. Simultaneous determinations made on adrenal glands of the same animals after treatment and reported in another communication(9), show depletions of ascorbic acid in adrenals as follows: normal, 38%; hypothyroid, 75%; and hyperthyroid, 31%. This can be compared to the preputial ascorbic acid depletion of: normal, 27%; hypothyroid, 89%; and hyperthyroid, 44%. It is apparent that in hypothyroidism a much greater depletion of ascorbic acid occurs in response to severe stress(10). This phenomenon of ascorbic acid depletion may be a generalized vitamin depletion, or it may be associated with steroid production. Histochemical studies of Montagna and Noback

(4) locate ascorbic acid in the cytoplasm of acinar cells, along with sudanophilic lipids. Since the aged sebum contains steroids, the preputial glands may be a more favorable site for elucidation of the relationship between ascorbic acid and steroid formation than the more complex adrenal glands.

The preputial content of ascorbic acid is lower in rats hypophysectomized 24 hours previously than in controls. This may be a result of depletion due to trauma associated with the operation without return to the normal level, or may be a result of decreased pituitary hormones.

Summary. 1. The normal level of ascorbic acid in the preputial glands of the young male rat is 60 mg %. 2. Adrenalectomy (3 wks) induces preputial gland hypertrophy, which is partially prevented by DCA. 3. Severe stress produces a depletion of preputial ascorbic acid, which is accentuated in hypothyroid animals. 4. Hypophysectomized rats (24 hr) have a level of preputial ascorbic acid which is 78% that of controls.

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Metabolism of Renal Cortex in Nephrotic Syndrome of Rats.* (19356)

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The chronic renal disease induced in rats by the intravenous injection of nephrotoxic sera obtained from rabbits(1) simulates the nephrotic syndrome as it is observed in infants and children(2). The site of the antibody-antigen reaction is in the renal cortex(3,4), probably in the basement membrane of the glomerulus(5,6). The initial injury elicits a chronic disease that may continue for 12 to 15 months or longer, without further injection of nephrotoxic antibodies.

It is impossible that heteronephrotoxins may have this prolonged effect by virtue of their continued presence. It appears more likely that the initial antibody-antigen reaction produces a cellular injury that may be irreversible. It thus seemed of interest to investigate whether this lesion is associated with metabolic alterations in the renal cortex of rats in which this disease had been induced.

Methods. Rats of the Long-Evans and Whelan strains were used. They were kept on Friskies and water. Renal disease was induced according to the technic previously described(2) when they were 4 to 5 weeks of age. Animals were chosen that had a well established disease for various lengths of time. Fourteen had had renal disease for less than 8 days, 5 for 1-4 weeks, 14 for 1-6 months, and 9 for 6-14 months. They were sacrificed by decapitation, blood being obtained for chemistry studies. One-fourth to one-half of each kidney was preserved for histological examinations. Immediately thereafter the remaining kidneys were freed of non-cortical tissue and placed in ice-cold Krebs-Ringer

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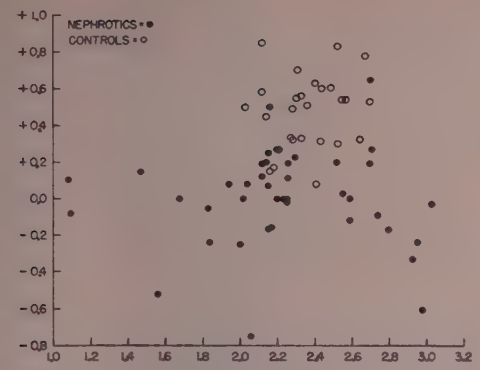

FIG. 1. Oxygen uptake of renal cortex slices from control and nephrotic rats. Abscissa: Qo_2 in Krebs-Ringer solution. Ordinate: Changes in Qo_2 produced by addition of glucose. The circles (○) represent the values obtained with controls, the dots (•) those with nephrotic animals.

solution(7). Slices 0.4 to 0.5 mm thick were prepared with a double blade tissue slicer. They were blotted with No. 50 Whatman filter paper, weighed on a torsion balance and placed in Warburg respirometer vessels (total volume 4 to 5 ml) containing 0.8 ml of Krebs-Ringer solution. Their respiration was measured in an atmosphere of 100% oxygen for a period of 90 minutes at 38°C, by the conventional Warburg technic(7). The time interval between decapitation and the initial manometric reading varied between 30 and 45 minutes. All solutions of substrates were prepared immediately before each experiment and were used in a final concentration of 0.02 molar.

Results. In the absence of substrate the average oxygen uptake of cortex slices of nephrotic rats did not differ significantly from that of the controls (Table I). In 29 control rats the average Qo_2 was 2.39 as compared to 2.28 obtained in 42 nephrotic animals. It should be pointed out, however, that in the experimental group the oxygen uptake varied within a wider range than in the control group (Fig. 1).

The addition of glucose invariably resulted in an increase in the oxygen uptake in the control slices, varying from +3 to +40%. The average value obtained from 27 animals was +22%. No consistent increase of the oxygen uptake after addition of glucose was noted when the kidney slices were obtained from nephrotic rats (Fig. 1). In this group of 41 animals a rise in the oxygen uptake by glucose was noted in 16. In 13 there was a decrease varying between −5% and −37%. In the 12 remaining rats the differences in the oxygen uptake in the presence and in the absence of glucose were not greater than ±3%. In this group as a whole the average change in Qo_2 produced by glucose was less than +1%.

When fructose was used instead of glucose, a somewhat greater increase in the oxygen uptake of the controls was noted. It varied between +22% and +45%; the average value obtained from 8 rats was +31%. Again there was no consistent increase after addition of fructose to the kidney slices of nephrotic animals (Fig. 2). In 6 of 11 experiments an increase varying between +9% and 30% was observed. In 5 this substrate produced a decrease in Qo_2 varying between −4% and −16%. The average change obtained in 11 rats was +5%.

In contrast to the above 2 substrates, the increase in Qo_2 of the nephrotic slices produced by the addition of lactate, succinate, glutamate, acetoacetate and butyrate was equal to or only slightly less than in the corresponding control experiments. (Table I). The

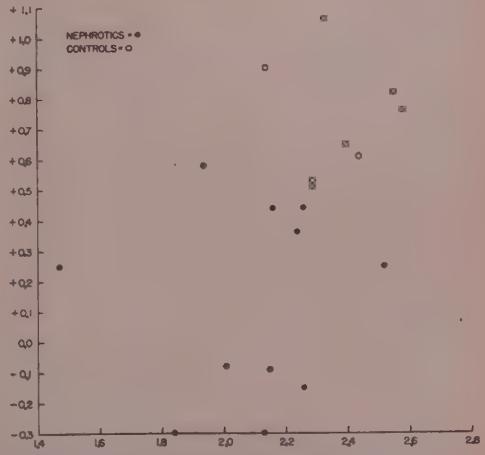

FIG. 2. Oxygen uptake of renal cortex slices from control and nephrotic rats. Abscissa: Qo_2 in Krebs-Ringer solution. Ordinate: Changes in Qo_2 produced by addition of fructose. The circles (○) represent the values obtained with controls, the dots (•) those with nephrotic animals.

TABLE I. Oxygen Uptake of Renal Cortex Slices from Control and Nephrotic Rats in the Presence and Absence of Various Substrates.

	No substrate		Na lactate		Na succinate		Na glutamate	
Rats	No.	Q_{O_2}	No.	Q_{O_2} % change	No.	Q_{O_2} % change	No.	Q_{O_2} % change
Control	29	2.39	11	+50	10	+57	9	+34
Nephrotic	42	2.28	19	+32	12	+63	12	+38
			Na acetoacetate		Na butyrate		Alanine	
Control			8	+31	8	+38	11	+68
Nephrotic			11	+24	11	+21	19	+33

TABLE II. Effect of Glucose, Lactate and Alanine on Oxygen Uptake of Liver Slices from Control and Nephrotic Rats.

	No substrate		Glucose	Na lactate	Alanine
Rats	No.	Q_{O_2}	Q_{O_2} % change	Q_{O_2} % change	Q_{O_2} % change
Control	6	1.26	+4	+34	+12
Nephrotic	9	1.24	+4	+27	+ 5

lower average figure observed with butyrate was due to the fact that this substrate did not increase the oxygen uptake in 2 of 11 experiments in which nephrotic kidney slices were used.

Alanine stimulated oxygen uptake of the slices to a lesser degree in the nephrotic group. In the latter an increase in Q_{O_2} equal to or higher than the lowest observed in the controls (+45%) was noted in only 7 of 19 experiments.

Similar experiments using glucose, sodium lactate and alanine as substrates were undertaken with liver slices of 6 control and 9 nephrotic rats (Table II). The oxygen uptake in Krebs-Ringer solution without substrate was the same for liver tissue obtained from healthy controls or nephrotic rats. It was, as is well known, about one half that of renal cortical tissue. There was no appreciable difference in the increase in oxygen uptake after addition of glucose and lactate by liver slices obtained from control or nephrotic animals. The diminished effect of alanine on the uptake of oxygen of liver slices obtained from nephrotic rats was due to the fact that 3 of the 9 experiments yielded values well below those obtained in the controls, while in the remaining 6 the values were within normal range.

Discussion. The data reported indicate that in the absence of added substrate the respiration of kidney slices from nephrotic rats is essentially unaltered. The oxidation of glucose and fructose, however, appears to be impaired. The reduction in oxidative utilization of glucose and of fructose cannot be the result of a depression of the cytochrome system, since oxidation of succinate by these slices was not affected. Furthermore, the increase in Q_{O_2} produced by this substrate in both normal and nephrotic slices was greater than that noted after the addition of glucose to the control slices. Since oxidation of lactate, succinate, and of glutamate appeared to proceed at a normal rate, the inability of glucose and fructose to raise the oxygen uptake of nephrotic kidney slices cannot be ascribed to an inhibition of the enzyme systems involved in the tricarboxylic acid cycle. Therefore this metabolic lesion must affect one or several reactions preceding the conversion of hexoses to lactic or pyruvic acid. Possibly this lesion may be the result of impaired phosphorylating mechanisms.

The observation that these two hexoses frequently reduced the oxygen uptage of nephrotic kidney slices below the level obtained without any substrate may indicate the oxidation of a different substrate. The oxidation of this substrate might be inhibited by hexoses possibly by a process of substrate competition.

Since in almost every experiment on addition of acetoacetate or of butyrate the same increase in the oxygen uptake was observed in normal and nephrotic kidney slices, no evidence is available indicating that fatty acid oxidation is impaired in the latter. Further studies using l- and d-alanine separately will be necessary for a proper interpretation of the results obtained with the racemic amino acid.

It should be pointed out that this metabolic lesion was independent of the duration of the renal disease, of the degree of the proteinuria, of the degree of hypertension, and of the severity of the histological lesion. The inability of glucose or fructose to increase the oxygen uptake was noted as early as 24 hours after the injection of heteronephrotoxins. It was also observed in rats which had the renal disease for 14 months with a normal or a high systolic blood pressure.

It has been shown(8) that heteronephrotoxins are rapidly and quite specifically adsorbed in the kidneys. In accordance with this mechanism the metabolic lesion was found in renal tissue but not in liver slices. It also has been reported(9) that the metabolism of renal cortex differs from the metabolism of renal medulla. Accordingly it is planned to extend the studies to medullar tissue, since there is evidence to support the view that the nephrotic syndrome affects the entire nephron even though the glomerulus is a primary site of the disease process(10,11).

Summary. 1. The oxygen uptake of renal cortex slices of 29 control rats and 42 animals in which the nephrotic syndrome had been induced by the intravenous injection of nephrotoxic sera was studied. 2. In Krebs-Ringer solution, without added substrates, the average oxygen uptake of cortex slices of nephrotic rats did not differ from that of the controls. 3. Addition of glucose or of fructose resulted invariably in an increase in the oxygen uptake in the control slices. No consistent increase after addition of these two hexoses was noted when the kidney slices were obtained from nephrotic rats. 4. The increase in oxygen uptake of nephrotic slices produced by lactate, succinate, glutamate, acetoacetate and butyrate was equal to or only slightly less than in the corresponding control experiments. 5. Racemic alanine stimulated the oxygen uptake of the slices to a lesser degree in the nephrotic group. 6. Since oxidation of lactate, of succinate, and of glutamate proceeded at a normal rate, the inability of glucose and fructose to raise the oxygen uptake of nephrotic kidney slices cannot be ascribed to an inhibition of the cytochrome system or of the enzymes involved in the tricarboxylic acid cycle.

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Complement Fixation with Brunhilde and Lansing Poliomyelitis Viruses Propagated in Tissue Culture.* (19357)

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It has recently been demonstrated that the complement fixation reaction can be used for the detection of antibodies against the Lansing type of poliomyelitis virus. Suspensions of infected tissues of the central nervous system, from adult(1) and infant(2) mice and cotton rats(3-5) were used as sources of antigen. Such starting materials inherently have several disadvantages, however, since they require, because of their high content of tissue components, cumbersome or time-consuming methods of purification, such as lipoid extraction, ultracentrifugation or methanol precipitation before they may be employed as antigens. Moreover, only the Lansing-like strains that can be propagated in these rodents have so far proved capable of fixing complement.

Tissue culture technics have provided a means of obtaining large quantities of virus-containing fluid that are relatively free of contaminating proteins and other tissue derivatives(6). Accordingly, an investigation was undertaken to determine whether these materials might provide antigens for use in a practical complement fixation test. The results so far obtained are presented.

Preparation of antigens. Supernatant fluids from tissue cultures infected with either the Lansing or the Brunhilde strain were used as starting materials for the concentration procedure. The virus was grown in suspended cell cultures(6) or roller cultures (tubes or 250 ml centrifuge bottles(7)). A control antigen was prepared in the same way from pooled non-infected control cultures included in the same experiments. Human tissue was employed. It consisted of either embryonic skin and muscle, embryonic intestine, adult uterus, or kidney. The latter was obtained from operations for hydrocephalus(8) in small children, or, in some instances, removed at the autopsy of children a few hours after death. Usually the supernatant fluids were pooled from several experiments involving different tissues from several embryos and children. It is logical to assume that the starting material for the preparation of antigen should contain a high concentration of virus and should be as free as possible of other substances. The supernatants from suspended cell cultures in which an ultrafiltrable medium is used(9), fulfill the second requirement, but in our experience usually contain less virus (Lansing virus; mouse titers ranging from 10^{-3} to 10^{-4} calculated per ml fluid) than roller cultures (mouse titers ranging from 10^{-4} to $10^{-5.5}$). The roller medium, however, usually contains the complex entities, embryo extract and horse serum. Recently the advantages afforded by both methods have been retained by the use of roller cultures in which the complex medium used as routine in such cultures is replaced by the simple medium after a satisfactory outgrowth of cells has occurred and after a large quantity of virus has been introduced as inoculum.

The ultrafiltration method described by Seibert(10) was applied to the concentration of the antigen. Alundum candles, impregnated with a 7% collodion solution in glacial acetic acid and washed, provided the filters. The filter candle was placed in a pharmaceutical graduate with a narrow bottom containing the tissue culture fluid. The filterable material was then removed through the filter by suction. In this way the fluid was almost completely removed. The non-filterable residue on the surface of the filter and at the bottom of the graduate was washed twice with 200 ml of the veronal buffer employed in the complement fixation test using the same procedure. After the last washing the vacuum was broken and the filter-outlet connected to a flask containing buffer and

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placed about 50 cm higher than the filter. The flow of fluid was thus reversed, washing the nonfilterable material off the surface of the filter. When a volume of 10-20 ml was attained it was again removed by suction. Finally, the nonfilterable material was taken up in about 2 ml of fluid passed in the reverse direction through the filter. This concentrate was clarified by centrifugation at 13,000 rpm for 15 minutes in a Servall SS1 centrifuge. All these procedures were carried out at about 4°C. The preparations have so far been concentrated from 60 to 600 times. Should drying of the virus occur during the ultrafiltration procedure no harmful effect may be anticipated since it has been determined that drying in air even at room temperature does not impair the antigenic activity of the concentrate. The resulting product, when derived from cultures containing the simpler medium, was clear or slightly opalescent, colorless or brownish, and slightly viscous when concentrated 600 times. On the other hand a similar preparation concentrated 60 times from fluids containing 5% horse serum and 10% beef embryo extract was very viscous. However, it could be used in the complement fixation tests. The concentrated antigens were frozen and stored at —20°C.

Antisera. Sera from monkeys and from human beings were tested. All were stored at —15 to —20°C. Monkey antisera against Lansing, Brunhilde and Leon virus, respectively, were kindly supplied by Dr. Jonas Salk. They were prepared by pooling sera from monkeys immunized with infected monkey cord together with adjuvant(11). Certain experiments, however, were carried out with the paired sera of a rhesus monkey before and after hyperimmunization in this laboratory with Lansing monkey cord without adjuvant. The Brunhilde serum had a 50% neutralizing endpoint of 1:640 against 100 50% paralyzing doses of Brunhilde virus(12). These sera, as well as many other monkey sera examined were found to be more or less anticomplementary in low dilutions. The anticomplementary effect was eliminated or markedly reduced in the following manner. The sera were diluted 1/2.5 in veronal buffer, heated at 60°C for 20 minutes, cooled in ice water and an equal volume of 10% crystalline bovine albumin added. The mixture was heated again at 60°C for 20 minutes. No significant difference was observed in the antibody titers (1/128) of a specimen of Brunhilde serum heated in this manner and another diluted ½ in veronal buffer and heated once at 56°C for 30 minutes. Paired sera were tested from poliomyelitis patients from whom virus had been isolated in tissue culture(13). Sera from 6 cases in the Brunhilde epidemic which occurred in Boston in 1949 were used together with the sera of 6 patients from the predominantly Leon epidemic of 1950. These sera diluted ½ in buffer were inactivated at 56°C for 30 minutes. Thereafter none of them proved to be anticomplementary to a significant degree.

Technic of the complement fixation test. The drop method described by Fulton and Dumbell(14) was adopted. It provides a simple and reliable technic and is especially valuable because of the small amounts of reagent needed. The following modifications that we have introduced should be mentioned: The preparation of glass dropping pipettes is laborious and they are easily broken. Accordingly we employed a No. 19 needle from which the beveled tip had been removed. It was fitted to a 1 ml Yale syringe from which the upper flange had been cut off to accommodate more readily a rubber bulb. Using distilled water this device delivers 20 mm^3 per drop.

TABLE I. Summary of Attempts to Remove Anticomplementary Activity of the Antigen.

Complement incubated in presence of:*	Complement titer
Veronal buffer	300
Untreated antigen†	14
Ether extracted antigen‡	20
Suspension of ether extract‡	300
5% bovine albumin	400
Antigen + 5% albumin	14
Antigen, 60°C 30 min	400

* Serial dilutions of complement were incubated overnight at 0°C.

† Antigen made from Lansing-infected roller bottles with embryonic skin-muscle; in this experiment with a concentration factor of 28 times.

‡ .3 ml of antigen extracted three times with 2 ml ether at 0°C. .3 ml of veronal buffer added to ether phase. Ether removed by evaporation *in vacuo*.

TABLE II. Effect of Heat on Anticomplementary and Antigenic Activity.

Lansing antigen* heated at	No serum	Monkey sera, diluted 1:5				
		Normal	Lansing 1	Lansing 2	Brunhilde	Leon
60°C 30 min	†	†	±	—	—	—
56°C	†	†	±	+	†	†
54°C	‡	—	±	+	‡	‡
52°C	+	—	+	—	—	—
50°C	+	—	+	—	—	—
No antigen		†	†	†	†	†

* Antigen concentrated 600 times from a pool of Lansing-infected suspended cell culture fluids.

† Complete hemolysis.

‡ Trace of unhemolyzed cells.

— = Not done. Serum Lansing 1 from same monkey as normal serum but after hyperimmunization (monkey cord without adjuvant). 2.5 units of complement added.

TABLE III. Complement Fixation Tests with Lansing and Brunhilde Antigens and Three Types of Monkey Antisera.

Antigen (55°C 30 min)	Titers of monkey sera		
	Lansing	Brunhilde	Leon
Lansing*	20	<5	<5
Brunhilde†	<5	≥40	<5
Normal‡	<5	<5	<5

* Same as in Table II.

† Concentrated 270 times from a pool of Brunhilde-infected suspended cell culture fluids.

‡ Concentrated 600 times from the control cultures carried in parallel with cultures infected with Lansing and Brunhilde viruses.

3 units of complement added.

The relatively small number of tests included in the present experiments made it unnecessary to employ the elaborate rack and boxes described by Fulton and Dumbell. Instead, enameled metal boxes were used. Two boxes were employed: one was kept at 37°C and the other in the ice box. The bottoms were covered with a layer of moist absorbent cotton. During the test ice cubes were placed in the bottom of the cold box. One to three sheets of lucite (9″ x 11″ x ⅛″) were supported by rubber stoppers in these boxes which were covered tightly with a sheet of "Parafilm."‡ The concentration of sheep's red cells (0.4%) was twice that used by Fulton and Dumbell. This modification gave clearer readings, but probably somewhat reduced the sensitivity. The same batch of pooled guinea pig serum was used throughout. This was stored in sealed ampules in the CO_2 box. Most of the titrations of antibody were carried out in the conventional manner, employing constant quantities of antigen and complement and increasing dilutions of serum. The complement titer which was assumed to remain essentially unchanged was, however, verified by titration performed contemporaneously with each test. The amounts of complement used in the test were thus found to vary between 2 and 3 units as indicated in the tables. The unit of complement was taken as the least amount of guinea pig serum that caused complete lysis of the cells. All necessary controls were included. The antibody titer is expressed as the highest dilution of serum giving about 50% or more inhibition.

Elimination of anticomplementary effect of antigens. The antigens, as well as the original tissue culture fluids, proved to be anticomplementary. Although an indication of specific complement fixation was observed with the Lansing antigens that were the least anticomplementary, it was obvious that this effect had to be minimized or abolished if conclusive results were to be expected. In attempts to accomplish this, the procedures mentioned in Table I were followed. It is evident that extraction with ether or addition of albumin were without effect, whereas heat treatment was completely effective. The results presented in Table II indicate that a temperature of 54-56°C for 30 minutes was necessary to abolish the anticomplementary activity. Within the temperature range 54-60°C, however, the capacity of the antigen to fix complement in the presence of homologous antibody was not abolished. Heat treatment (55-56°C for 30 minutes) has so far been

‡ Manufactured by Marathon Corp., Menasha, Wisc.

TABLE IV. "Box Titration" of Brunhilde Antigen with Homologous Monkey Antiserum.

Dilution of antigen	Dilution of antiserum								
	4	8	16	32	64	128	256	512	No serum
2	—	+	+	+	+	+ to ±	†	†	†
4	+	+	+	+	+	+	†	†	†
8	+	+	+	+	+	+	±	†	†
16	+	+	+	+	+	+	+ to ±	†	—
32	+	+	+	+	+	+ to ±	±	†	—
64	+	+	+	±	±	†	†	†	—
128	+	±	‡	‡	†	†	†	†	—
No antigen	+ to ±	†	†	†	—	—	—	—	—

† Complete hemolysis. ‡ Trace of unhemolyzed cells. — = Not done.
Same antigen and antiserum as mentioned in Table III. 2 units of complement added.

TABLE V. Complement Fixation Tests on Sera from Cases of Poliomyelitis.

Name	Age, yr	Type of case	Type of virus isolated	Time after onset, days	Serum titer	
					Brunhilde antigen*	Cont. antigen†
F.	6 6/12	Paralytic	Brunhilde‡	8	16	—
				54	16	<2
D.	5 2/12	"	" ‡	8	<2	—
				16	8	<4
				440	<2	2
Du.	10 11/12	"	"	5	2	—
				19	16	<2
J.	2 10/12	"	"	4	16	—
				22	32	<2
Fa.	6 5/12	Nonparalytic	"	5	2	—
				26	<2	<2
I.	9 9/12	Paralytic	"	7?	<2	—
				29	16	<2
L.	11 6/12	"	"	4?	<2	—
				28	4	<2
S.	5 5/12	"	Leon	2	<4	—
				19	<2	2
M.	3	"	"	2	<2	—
				29	<2	<2
K.	1 2/12	"	"	5	<2	—
				30	<2	<2
Ma.	10 11/12	Nonparalytic	"	2	≥8	—
				22	16	2
R.	4 11/12	Paralytic	"	5	8	—
				25	4	<2

* Same as in Table III; diluted 1/10.
† " " " " " 1/4.
‡ Brunhilde-like virus isolated from a pool of stools from two siblings F. and D. 2-2.5 units of complement added.

effective in the case of 4 different batches of antigen (Lansing, Brunhilde and normal control antigens) concentrated from 110 to 600 times. This procedure also eliminated the anticomplementary effect of crude tissue culture fluids. The heated antigens could be stored at —20°C and repeatedly thawed and frozen without changing their qualities.

Specificity of the reaction. A. Monkey sera. The specificity of Lansing and Brunhilde antigens is clearly illustrated by the results recorded in Table III. A "box titration" (Table IV) of the Brunhilde antigen against the homologous monkey hyperimmune serum revealed an optimal antigenic activity at the dilution 1/16. This corresponds to about a 20-fold concentration of the antigen present in the crude tissue culture fluid.

B. Human sera. Acute and convalescent serum specimens from 12 cases of poliomyelitis were tested for antibodies against the Brunhilde antigen. The convalescent specimens were in addition tested against the control antigen. The results are shown in Table V. Of the 7 patients yielding Brunhilde-like viruses, 6 had complement fixing antibodies and 4 developed a significant rise (at least 4-fold) in titer during convalescence. In the sera of 3 of these patients an increase in antibody was not demonstrated. In 2 of them, however, relatively high titers were characteristic of both serum specimens—a finding which suggests that the maximum response had occurred by the time the first specimen was taken. In view of our limited experience no entirely satisfactory explanation can now be given for the failure of the third patient (Fa.) to develop antibody. It might be mentioned, however, that this patient, as well as J. and L., had been treated with ACTH during the acute phase. The results afford some evidence that antibody tends to develop early in the disease. How long it may persist in detectible quantities must await further study, but the findings with the sera of patient D. suggest that in some individuals this period may not be long.§ None of the sera mentioned in Table V reacted significantly with the control antigen. Moreover, the few insignificant reactions noted usually did not correlate with the reactions with the virus antigen.

The sera of 2 of the 5 patients with Leon-like virus fixed complement with the Brunhilde antigen. No evidence of increase in titer, however, was observed. The significance of these antibodies is as yet obscure. The following possibilities to account for their presence come to mind: a) they were the result of a previous infection with Brunhilde-like virus; b) infection with a Leon-like virus in human beings may elicit antibodies which cross react with Brunhilde virus; c) these patients may have undergone a mixed infection with both viruses, although only Leon-like viruses were isolated. In one of the patients (Ma.) from whom a Leon-like virus was isolated but in whom no increase in antibody was demonstrated, the acute phase specimen was obtained within 2 days after the onset. This circumstance renders hypothesis b) and c) less probable.

Summary. Antigens which gave specific complement fixation were concentrated from the supernatant fluids of tissue cultures infected with poliomyelitis viruses (Lansing and Brunhilde strains) by a method of ultrafiltration. Their anticomplementary activity was abolished by heating. The drop method of Fulton and Dumbell was used and offered a practical way of performing the tests with minimal amounts of antigen. Specific antibodies were demonstrated in the sera of hyperimmunized monkeys as well as in sera of poliomyelitis patients.

§ Since this paper was submitted for publication additional specimens of sera taken 1.5-2.5 years after the illness have been obtained from all patients with antibodies against Brunhilde except patient I. When tested at the same time as the earlier specimens they all exhibited a marked drop in complement fixing antibody.

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Influence of Diet on Stability of Rat Erythrocytes. (19358)

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The structural stability of the mammalian erythrocyte is in part dependent upon the state of order among the components which constitute its surface(1). This stability resides in the quantitative and spatial relationships of lipids and proteins forming the cell membrane, which is built up as a tricomplex system due to the ionic polarity of the specific lipids and proteins. It has been shown by hemolytic experiments that calcium may enter the membrane structure and considerably enhance its stability through more powerful ionogenic forces(2). Variations in other components of the complex may theoretically alter the stability, although such changes have not been accomplished experimentally.

Alterations in the lipids and proteins of erythrocytes in anemias were observed by Erickson and her colleagues(3). It is perhaps significant that in hemolytic and hypochromic anemias the quantity of total lipid and especially the proportions of the phospholipids, acid soluble lipid and cholesterol were decreased. The deviations in these two diseases suggest that it may be possible to initiate similar changes experimentally and alter the stability of the cell. In the present studies dietetic conditions which affect the osmotic stability of the rat erythrocyte have been examined. The effect of alloxan, in view of its *in vivo* hemolytic action, was also investigated.

Materials and methods. Male Sprague-Dawley albino rats, weighing 200 g, were placed without restriction for a period of 3 months on the diets indicated in Table I. One hundred rats were maintained on the pellets, and 2 groups of 48 rats on each of the synthetic diets. Samples of blood, collected from the tails into dried tubes containing 0.2 mg of sodium oxalate, were suspended as 0.01 ml aliquots (by Breeder-Brew pipette) in 2.0 ml volumes of various concentrations either of saline or of a solution which contained 9 mg % of alloxan monohydrate (Eastman Kodak No. 1722) dissolved in either 0.5% or 1.0% sodium chloride. The extent of hemolysis was estimated spectrophotometrically with a Coleman Spectrophotometer No. 14 by the technic of Hunter(4), and the percentage hemolysis was so calculated as to determine the cumulative curve or ogive by Bolton's procedure(5). Two or more analyses were made on each rat so that each point of the curve represented the averages of between 96 and 200 separate analyses.

TABLE I. Composition of Diets.

Synthetic	
Sucrose	710 g
Casein (vit. test)	210
Salt mixture 4(6)	40
Cystine	2
Choline	1
Ca. pentothenate	20 mg
Niacin	10
Riboflavin	3
Pyridoxin	2.5
Inositol	100
Biotin	.1
Folic acid	.2
Thiamin	2
Halibut liver oil	2 drops/wk
Fat*	—
Pellet (master mix rabbit pellets)†	
	%
Ground wheat	.5
Irradiated yeast	.9
Ground limestone	.9
Defluorinated phosphate	.3
Salt content:	
Manganese sulfate	.003
Potassium iodide	.0028
Iron oxide	.00032
Copper sulfate	.00026
Cobalt sulfate	.00026
Alfalfa meal	Amt unspecified
Ground barley	" "
Beet pulp	" "
Linseed oil meal	" "
Ground corn meal	" "
Molasses	" "
Soybean oil meal	" "
Pulverized oats	" "

* To constitute the final high fat diet either lard or "spry" was melted and mixed with above mixture, so that each 100 g of final diet contained 20 g of fat.

† This pellet diet, as obtained from McMillen Feed Mills, Fort Wayne, Ind., is specified to contain 2% crude fat and 18% protein.

Results. The behavior of the erythrocytes

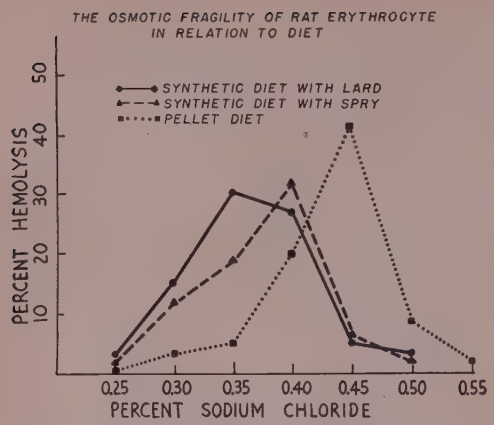

FIG. 1. The red cells were suspended in sodium chloride solutions of varying tonicity. Extent of hemolysis is estimated by determination of hemoglobin released after constant time of 30 min.

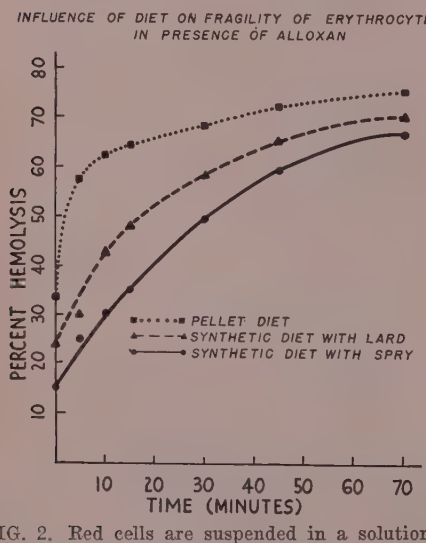

FIG. 2. Red cells are suspended in a solution of .5% NaCl containing 9 mg % alloxan. Extent of hemolysis is estimated by determination of hemoglobin released at staggered intervals up to 70 min.

from the 3 dietetic groups is depicted in Fig. 1, which demonstrates the considerable instability of the cells in animals on the pellet diet as compared with cells of rats on synthetic rations. The cells from animals on the two synthetic diets differ only slightly, with a slightly greater stability in those consuming lard. Microscopic examination of the cells substantiates the results observed in the fragility study. The cells from "pellet" fed rats pass rapidly through the stages of crenated disc, crenated sphere and prolytic sphere, whereas these changes are relatively slow in rats sustained on the synthetic diets. The red cell counts and the hemoglobin concentrations were not significantly altered in any of the groups.

In all groups the extent of hemolysis in 0.5% sodium chloride alone is insignificant. When alloxan is present in the suspending fluid (9.0 mg %) the cells of all groups are rapidly lysed (Fig. 2). The rate of hemolysis is most rapid in the cells from "pellet" fed animals and less in those of the other "synthetic" groups, which differ only slightly. When 1% sodium chloride is used no hemolysis occurs in the presence of alloxan, indicating a connection between pre-hemolytic swelling and the hemolytic action of alloxan on the cells.

Discussion. The stability of the erythrocyte of the albino rat is affected by alterations in diet. This is manifested by a change in osmotic fragility and an instability in the presence of alloxan. The relationship between the preconditioning of the cells by a non-hemolytic hypotonic level of saline prior to the action of alloxan is unique. It may be related to the conversion of alloxan into dialuric acid necessary for hemolysis by the compound(7); the prehemolytic swelling of the erythrocyte in the 0.5% sodium chloride allows the alloxan to penetrate the cell where, by interaction with glutathione(8), it is converted into the hemolytically active dialuric acid. This is further supported by the fact that the most easily lysed cells are those (pellet) which swell most readily in the hypo-osmotic solution.

Summary. The stability of the erythrocytes of albino rats is affected by alterations in diet. This change is reflected in both the osmotic fragility and is related to the rate of hemolysis in the presence of alloxan.

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Electron Microscope Studies on the Ciliary Apparatus of the Gill Cells of *Mya arenaria.** (19359)

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The nature of the ciliary apparatus has long been a subject of discussion. For reviews and literature pertaining to this subject consult Engelmann(1), Saguchi(2), Grave and Schmitt(3) and Worley(4). Morphology of the ciliary apparatus becomes especially significant in the formulation of theories concerning the physiology of ciliary movement (5). Clear pictures have been drawn of certain fixed ciliated cells showing the cilia, basal bodies and intracellular fibrils which often converge, forming a cone-like structure with its apex at one side of the nucleus(1). Because of the fact that the intracellular fibrils cannot be easily seen in the living cell with the light microscope, they have been thought of as artifacts by some (see discussion in 3).

The purpose of this report is to present electron micrographs of cells from the laterofrontal and frontal regions of the gill filaments of *Mya arenaria*, showing cilia, membranes, basal bodies, and intracellular fibrils.

Methods. Material used in this study was fixed in Regaud's solution and sectioned at 0.2 μ according to the method of Beams, *et al.* (6). The electron microscope used was an R. C. A. model EMU-2B, equipped with an unbiased gun. Magnification of the electron micrographs is indicated by the 1 μ scale drawn on Fig. 1.

Results. The cilia of laterofrontal cells (a, Fig. 1 and 3) appear to be compound *i.e.*, consisting of 3 to 4 filaments of approximately 250 Å in diameter. However, extensive fraying of them into the individual fibrils was not observed as has been demonstrated for the cilia of *Paramecium* and filaments of sperm tails (unpublished). At their bases the cilia are spaced only about 500 Å apart. This is close as compared to the distance between the cilia of the frontal cells (Fig. 2). The bodies of the cilia seem to be in many instances in contact with each other. However, no mucous-like substance which might function to hold them together as a unit was observed. This is mentioned in view of the findings of Carter (7) who observed that in the laterofrontal cells of *Mytilus* all the elements of the vibratory apparatus of a cell beat as a unit or "cilium", comparable to the cirri of certain protozoa(8).

Short extracellular filaments (Fig. 3) are thought to be broken cilia. Some evidence for a cuticular border consisting of short filamentous processes is shown at upper left in Fig. 1. However, it cannot be determined for certain whether or not these filaments are continuous all the way across the border. In the frontal cells, definite cuticular processes of about 400 Å in diameter and 8300 Å in length are sharply defined (g, Fig. 2). These are in many ways comparable to those seen in the kidney tubules and certain intestinal cells (9,10). Only the proximal portions of the cilia are shown in Fig. 2; they are more widely spaced than in the laterofrontal cells

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FIG. 1. Montage of electron micrographs of laterofrontal gill cell of *Mya.* Cilia, a; surface cell membrane, b; basal bodies, c; intracellular fibrils, d; and lateral cell membrane, e; are demonstrated.

FIG. 2. Electron micrograph of the distal portion of a frontal gill cell of *Mya,* showing proximal portions of cilia, f; cuticular border with filamentous processes, g; surface membrane, h; basal bodies, i; and intracellular fibrils j.

FIG. 3. Same as in Fig. 1 except a more oblique section of the cell.

(Fig. 1 and 3), and are too opaque to determine whether or not they are made up of smaller fibrils. At b, (Fig. 1 and 3) and h, (Fig. 2) appears a continuous electron opaque line which probably is the cell membrane. The only other possible interpretation is that it represents a kind of neuroneme similar to that described for *Paramecium* (11). In some areas of the cell this structure appears double (center part of Fig. 1).

The basal bodies are shown at c, (Fig. 1 and 3) and at i, (Fig. 2). They are fused with, and are slightly larger than, the bases of the cilia. In addition, they show a greater electron density than do the cilia. Fused also with the basal bodies and extending basically in the cytoplasm are the intracellular fibrils (d, Fig. 1 and 3; j, Fig. 2). There is a single intracellular fibril attached to each basal body and associated cilium. The intracellular fibrils converge, forming a conelike structure with its apex at one side of the nucleus (Fig. 1). We were not able to determine whether or not the apex of the cone is embedded in any specialized body of the cytoplasm. In certain preparations the side of the cone seems to be in contact with the nuclear membrane (Fig. 1). In Fig. 2 the section is at such an angle that the outline of the cone is not clear. The intracellular fibrils are approximately 1000 Å in diameter. Details of their internal structure could not be determined. Furthermore, we were not able to determine whether or not lateral connections extend between them as reported by Worley(12). In places strands appear to connect the individual fibrils, but this may only represent coagulated protoplasm. At e (Fig. 3) is seen the membrane of the cell.

Discussion. Evidence here presented substantiates the view that a single cilium, basal body and intracellular fibril are morphologically united and conceivably constitute the functional unit of the ciliary apparatus in these cells. The reality of the intracellular fibrils is not questioned. Their sharp definition and consistency in number and appearance argue for their presence in the living cell. Other evidence to support this view is that they are birefringent(13) and have recently been seen in the living cell by use of the phase-contrast microscope(12). A review of the literature on ciliated cells indicates that the basal bodies are probably always present. However, the intracellular fibrils have only been demonstrated in certain of the large ciliated cells of invertebrates. Whether or not they function in the mechanism of regulating cilia beat or are only supporting structures in these cells is unknown.

Summary. The electron microscope demonstrates the morphology of the ciliary apparatus of the laterofrontal and frontal gill filament cells of *Mya* to be composed of cilia which appear to be made up of smaller fibrils, basal bodies which are fused with the basal ends of the cilia and intracellular fibrils which are also fused with the basal bodies at their distal ends. These intracellular fibrils extend basally and converge forming a cone-like structure with its apex ending in a region near the side of the nucleus. Frontal cells of the gills show in addition to the above components, a striated cuticular border composed of short filaments approximately 8300 Å in length and 400 Å in width. The cilia of the laterofrontal gill cells are more closely spaced than are those of the frontal gill cells.

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Sulfhydryl Variation in Bacterial Enzymes in Relationship to Chemotherapy with the Nitro-Furans.* (19360)

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The nitro-furans and furacin (5-nitro-2-furaldehyde semicarbazone) inhibit the growth of many bacteria(1), at least one filterable virus(2) and certain tumors(3). Recently it has been reported that furacin has the properties of a limited sulfhydryl (SH) inhibitor (4). In a further investigation of the relationship of furacin to SH enzymes in bacteria, furacin-resistant bacteria were found resistant to such SH inhibitors as p-chloromercuribenzoate (pCMB) and trivalent arsenicals. It was also observed that bacterial urease from *Micrococcus pyogenes var. aureus* was inhibited by SH inhibitors when the organisms were grown in media containing urea. When urea was omitted from the media, ureases were produced that were not inhibited by SH inhibitors or by furacin.

Methods. The basal medium for all experiments contained 2.5 g of peptone (Difco) and 1.5 g of beef extract (Difco) per liter. Four different laboratory strains of *Micrococcus pyogenes var. aureus* were tested. The inoculum (0.1 ml per liter) was prepared from a 24-hour culture grown in double strength basal medium. The inhibition experiments were carried out with organisms grown in basal medium alone, basal medium plus 2% urea and basal medium plus 1% glucose. The organisms were harvested after 14 hours and washed 3 times with small quantities of M/15 phosphate buffer pH 7.4. The weight of organisms was determined by turbidity measurements against a standard curve. Urease activity was determined by aeration and Nesslerisation in accordance with a procedure already described(4). The results of some typical experiments using whole organisms as the source of urease with various inhibitors are given in Table I. The cells were exposed to the inhibitor for 15 minutes, the urea substrate was then added and the reaction allowed to proceed for an additional 15 minutes, after which it was stopped with 1N HCl. In reactivation experiments, cysteine was added along with the substrate. The control experiments were identical in every respect except that water was substituted for the inhibitor.

Results. The total inhibition of urease activity with pCMB varied from 25% to 50% when the organisms were grown in the medium containing urea. However, no inhibition was noted when the organisms were grown in the absence of urea. The trivalent arsenicals showed the same pattern of inhibition as did furacin and pCMB. The urease of furacin-resistant organisms grown in the presence of urea was not inhibited by either pCMB or furacin. However, the same molar concentrations of the trivalent arsenicals p-carboxyphenylarsenoxide inhibited 6%, p-aminophenylarsenoxide 16%, and mapharsen 22% of the activity of urease derived from the furacin-resistant strain. Both the furacin and pCMB inhibitions of the susceptible organisms were reversed by cysteine (0.01 M). The furacin reactivation was 65% and the pCMB reactivation 82%.

Cell free extracts from organisms grown in the various media described above were prepared by the alumina method of Scott and Cohen(5). Results using such extracts are shown in Table I. The procedure used in measuring the activity of the cell free ureases was similar to the procedure with the whole cells. The cell free extracts behaved in exactly the same manner toward the inhibitors as the whole cell preparations. However, urease prepared from furacin-susceptible cells grown in the presence of urea was largely inactivated by the extraction procedure. A similar preparation from resistant cells was not inhibited.

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TABLE I. Effect of Various SH Inhibitors on Ureases from Whole Cell Preparations of *Micrococcus Pyogenes var. Aureus** and Cell Free Extracts from Organisms Grown in Various Media.

Source of urease	Inhibitor†	mg NH_3 nitrogen		% inhibition	
		Whole cells‡	Extract§	Whole cells	Extract
Basal medium + 2% urea	Furacin	.035	.014		
	—	.060	.019	41.6	‖
	pCMB¶	.029	.012		
	—	.060	.019	51.6	‖
	pCPA**	.034	—		
	—	.066	—	49.5	—
	pAPA††	.031	—		
	—	.066	—	53	—
	Mapharsen‡‡	.029	—		
	—	.066	—	56	—
Basal medium alone	Furacin	.044	.043		
	—	.043	.045	0	0
	pCMB	.043	.044		
	—	.043	.045	0	0
Basal medium + 1% glucose	Furacin	.087	.072		
	—	.088	.072	0	0
	pCMB	.086	.071		
	—	.087	.073	0	0

* Furacin susceptible cells used.
† All inhibitors 7×10^{-4} M.
‡ 4 mg washed cells used per tube.
§ Extract equivalent to 2.5 mg of whole cells used per tube.
‖ Activity too low for reliable measurement of % inhibition.
¶ p-chloromercurybenzoate.
** p-carboxyphenylarsenoxide.
†† p-aminophenylarsenoxide.
‡‡ 3-amino-4-hydroxyphenylarsenoxide HCl.

Discussion. These experiments suggest that *Micrococcus pyogenes var. aureus* produces ureases with different affinities for SH inhibitors. In the absence of urea, a urease is produced which is less susceptible than jack bean urease to SH inhibitors(4). This may indicate that these ureases either do not contain SH groups or have SH groups that are slowly reactive or relatively inaccessible to SH inhibitors such as pCMB. Previous experiments with dehydrogenases(4) have shown that in the processes of the development of furacin resistance, SH dehydrogenases become resistant to pCMB and trivalent arsenicals. A similar phenomenon seems to occur with ureases from *Micrococcus pyogenes var. aureus,* although these organisms synthesize urease, which may not be dependent for its function on the presence of free SH groups when the bacteria are grown in media free of urea. These experiments also indicate that in bacteria the same enzyme may function either as a SH or non-SH enzyme. The exact nature of the transformation from SH to non-SH is open to conjecture. Two substrains may exist, one carrying the SH urease and the other the non-SH enzyme. This transformation may be the result of adaption or may be genetically controlled. Both enzymes may be simultaneously present in the same organism; the type of enzyme predominating in a given reaction may be dependent on the substrate.

Several theories have been advanced to explain the phenomenon of resistance to antibacterial agents such as alternate metabolic pathways, formation of antagonists or changes in permeability(6). It does not appear likely that the above result with bacterial ureases or the previous experiments with dehydrogenases showing cross-resistance with various SH inhibitors, can be explained solely on the basis of the above theories. The possibility of another mechanism should be considered in explaining resistance to the nitrofurans. Bacteria may be able to transform their enzymes into either the SH or non-SH form as circumstances may require. This phenomenon may be designated as SH shift. A definitive proof of the theory of SH shift will have to await the actual isolation from bacteria of the postulated SH and non-SH forms with a single

enzyme. The possibility should also be considered that changes can occur with other labile or reactive groups, such as free amino or hydroxy groups on the surface of enzyme proteins, in connection with other types of resistance, especially where the usual theories are not entirely applicable.

Results which are analogous to the postulated phenomenon of SH shift have been described. Ingbar and Kass(7) have shown that normal hemoglobin has two moles of SH per mole of hemoglobin while hemoglobin from sickle cell anemia has three moles of SH per mole. Hughes(8) has isolated two fractions of serum albumin, one fraction having a SH group and the other appearing to have no SH group. There are some instances where the same enzyme will function either in SH or in an apparent non-SH form depending on the tissue from which it is derived. The α-glycerophosphate dehydrogenase of rabbit thigh muscle is apparently non-SH and is not inhibited by furacin(4). The same enzyme from *E. coli* is inhibited by furacin(4). The presence or absence of a SH group in a given enzyme may not affect its ability to function on a given substrate. The presence of the SH group, however, may make the enzyme liable to inhibition by SH inhibitors.

Summary. In the presence of urea, *Micrococcus pyogenes var. aureus* produces a urease that is inhibited by furacin, p-chloromercuribenzoate and trivalent arsenicals. When the organisms are grown in the absence of urea or when furacin-resistant organisms are grown in media containing urea, ureases are obtained that are not affected by the above inhibitors. In explanation of these results as well as those previously reported involving the inhibition of dehydrogenases, it is suggested that some aspects of resistance to the nitrofurans can be explained by a shift of certain enzymes from the SH to a non-SH form.

The authors wish to thank Dr. L. A. Sweet of Parke, Davis and Company for the arsenicals and Drs. W. L. Stillman and L. Eugene Daily of the Eaton Laboratories for the furan derivatives used in this study.

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A Cofactor for the Formic Hydrogenlyase Enzyme System.* (19361)

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A survey of the literature concerning the effects of oleic acid on the growth of microorganisms reveals 3 major findings: 1) this fatty acid is essential for the optimal growth of some species of the genera *Lactobacillus*, *Corynebacterium*, and *Mycobacterium*(1-4); 2) oleic acid is required for certain strains of lactobacilli only in the absence of biotin which it apparently replaces(3,5); and 3) in sharp contrast to the growth stimulating effect, the toxic action of this fatty acid for various microorganisms has been recognized(2-4,6). These actions are not strictly limited to oleic acid, since some of these effects have been reported for other unsaturated fatty acids, chief-

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ly linoleic and linolenic. Recent studies designed to investigate the possible enzymatic functions of oleic acid, and the relationship of this fatty acid to biotin, have demonstrated that oleate is required for the optimal activity of the formic hydrogenlyase (FHL) and formic dehydrogenase enzyme systems(7).

The present paper reports further studies on the role of oleate in the FHL system and demonstrates the existence in certain natural materials of a substance more active than the fatty acid.

Materials and methods. The organisms employed were a biotinless mutant strain of *Escherichia coli* and a normal wild strain of *Aerobacter aerogenes* (D-1). The basal medium contained 0.2% each of KH_2PO_4, K_2HPO_4, NaCl, and $C_6H_5O_7Na_3 \cdot 2H_2O$; 0.4% $(NH_4)_2SO_4$; 0.5% acid-hydrolyzed vitamin-free casein; 0.1% each of $MgSO_4 \cdot 7H_2O$ and HCOOH; 0.01% $MnCl_2$; and 0.001% $Fe_2(SO_4)_3$. The medium was adjusted to pH 6.5 before autoclaving, inoculated with washed suspensions of the organism, and incubated at 30°C for 15-18 hours. The cells were harvested by centrifugation, washed once with H_2O, and resuspended in H_2O to give the desired cell concentration. *A. aerogenes* was grown in the basal medium, while the *E. coli* mutant was cultured in the basal medium containing 10^{-4} μg of biotin per ml. FHL was determined at 37°C in an atmosphere of N_2 by measuring H_2 production from formate manometrically. The Warburg flask contained KOH in the inner well to absorb the CO_2 produced; formate, phosphate buffer, water, and any other materials were placed in the main compartment; the cells were added to the side arm and were tipped into the main compartment after equilibration. When desired, formate disappearance was measured colorimetrically(8).

Results. The data plotted in Fig. 1 demonstrate two important points: 1) that cells grown in the presence of oleic acid have an active FHL enzyme system in contrast to cells grown without this fatty acid, and 2) that cells harvested from the medium containing biotin and exhibiting essentially no FHL activity were capable of being stimulated by

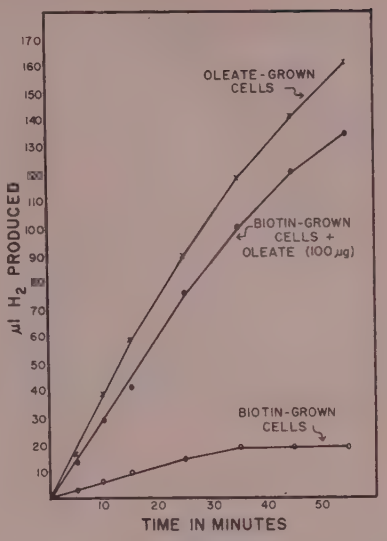

FIG. 1. Effect of oleic acid on the FHL activity of resting cell suspensions of *E. coli* (biotinless mutant). (Biotin-grown cells were harvested from the basal medium containing 10^{-4} μg biotin per ml; the oleate cells were grown in the basal medium plus 100 μg oleic acid per ml in place of biotin. Reaction run at 37°C in M/15 phosphate buffer pH 6. Cell concentration equivalent to .15 mg nitrogen. Formate added in M/10 conc. (.1 ml); final volume 3 ml).

oleate. These findings suggest that oleic acid, or some substance readily synthesized from it by the living cell, functions as a cofactor in this system.

The specificity of oleic acid was examined by studying the effects on the FHL system in resting cell suspensions of *E. coli* of each of 2 unsaturated fatty acids, linoleic and linolenic, and of several saturated fatty acids, stearic, palmitic, and pimelic. The experimental findings are presented in Table I. It may be seen that each of these substances was capable at times and at suitable concentrations of stimulating this system. The unsaturated fatty acids were more effective than the saturated members and oleic acid was the most active. On the other hand, acetate has been uniformly inactive. These data were interpreted to suggest that the living cell is capable, under certain conditions, of synthesizing a cofactor for the FHL enzyme from several fatty acids, and that of those tested oleic acid is the most suitable precursor.

TABLE I. Stimulation of FHL by Fatty Acids.*

Fatty acid	Concentration (μg) 100		10		1	
Oleic	13/15†	87‡	16/16	100	7/9	78
Linoleic	8/15	53	12/16	75	4/8	50
Linolenic	13/15	87	8/15	53	1/8	13
Stearic	7/14	50	2/15	13	1/7	14
Palmitic	9/15	60	4/16	25	1/6	17
Pimelic	5/15	33	2/15	13	1/8	13

* *E. coli* (biotinless mutant).
† Denominator = No. of experiments. Numerator = No. of positive experiments.
‡ % of experiments in which stimulation was recorded.

TABLE II. Comparative Activity of Oleate and Natural Materials.*

Additions	FHL activity†
0	27
Oleate, 10 μg	260
Yeast extract, 1 mg	960
0	0
Oleate, 100 μg	53
Yeast extract, 1 mg	940
0	550
Oleate, 50 μg	588
Yeast extract, 2 mg	1650
Liver " 2 mg	1120
0	150
Oleate, 100 μg	370
Yeast extract, 2 mg	610
Liver " 2 mg	550
0	350
Oleate, 50 μg	550
Yeast extract, 5 mg	1800
" " 1 mg	1400
" " .5 mg	950

* *E. coli* (biotinless mutant).
† Expressed as Q_{H_2} = $\frac{\mu l\ H_2 \text{ produced per hr}}{\text{mg cell N}}$.
Yeast and liver extracts are dried hot-water extracts.

Attempts were made to learn whether natural materials contain substances capable of stimulating this enzyme. That such substances exist in nature can be seen by examination of the data presented in Table II. Water extracts of yeast and liver were found to be more active than oleic acid. The organic nature of the stimulatory material was demonstrated in the following manner. The extracts were incinerated and the ash tested both by incorporating suitable amounts into the growth medium, and by the addition of the ash to the contents of the Warburg vessel. The results were uniformly negative.

Yeast extract was studied further in order to ascertain the stability of the stimulatory material. It was found that the activity of the yeast extract was increased greatly by hydrolysis at 121°C for 3 hours in 0.9 N H_2SO_4, and that hydrolysis at greater acid concentrations resulted in gradual destruction of this activity. Autoclaving without added acid resulted in a slight increase in activity. All samples were neutralized with $Ba(OH)_2$ before testing. The comparative activity of unhydrolyzed and hydrolyzed yeast extract may be seen by the titration plotted in Fig. 2.

Although suitable hydrolysis of yeast extract resulted in a marked increase in its activity with respect to the FHL enzyme system, it is emphasized that the unhydrolyzed material exhibited considerable activity. This observation suggested either of two possibilities: 1) that 2 factors are present in yeast, one available before hydrolysis, and the other after hydrolysis, or 2) that there is one factor and it is available only after hydrolysis, but the living cell is capable of accomplishing enzymatic hydrolysis. These possibilities were tested experimentally by studying dried cell preparations of *A. aerogenes* to learn the lability to the drying process of the enzyme(s) essential for hydrolysis of the yeast extract. The cells were dried by 2 methods, 1) acetone and 2) *in vacuo* over Drierite in a desiccator. The acetone-dried cells were inactive. The vacuum-dried preparations were uniformly capable of carrying out the breakdown of formate to CO_2 and H_2, thus establishing that the FHL enzyme system is stable to vacuum drying. The results given in Table III, when compared to the data plotted in Fig. 2, demonstrate that unhydrolyzed yeast extract was considerably less active when tested in the

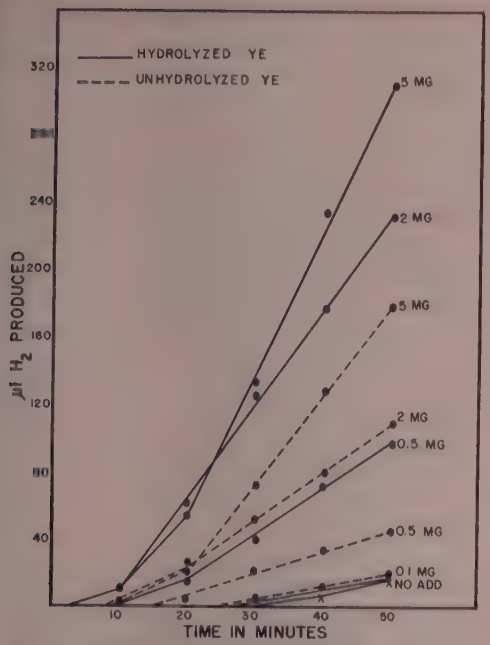

FIG. 2. Relative activity of hydrolyzed and unhydrolyzed yeast extract. (FHL activity in resting cell suspensions of *A. aerogenes*. Conditions of reaction as for Fig. 1 except .2 ml of M/10 formate added.)

dried preparation than in the living cell. In contrast to these findings, the acid-hydrolyzed extract maintained its ability to stimulate the FHL system. These results suggest that the cofactor present in yeast extract is released only after acid or enzymic hydrolysis, and that the degree of stimulation of the unhydrolyzed yeast extract is a reflection of the ability of the living cells to accomplish enzymic hydrolysis, an ability reduced greatly by vacuum drying of the cells. However, these results may also be interpreted to suggest that the dried preparations respond only to the hydrolyzed material because, in contrast to the unhydrolyzed yeast, it contains two factors. At present we are unable to distinguish between these two possibilities. It is suggested that the stimulatory material(s) present in natural substances are derived from a fatty acid precursor, possibly oleic acid.

Summary. 1. The formic hydrogenlyase enzyme system (FHL) is activated by oleic acid, thus representing an enzymatic function for a fatty acid. 2. Other fatty acids are capable of stimulating FHL activity in bacterial cell suspensions under certain conditions, but oleate is the most effective of those tested. 3. Yeast and liver contain a substance or substances more active than oleic acid. 4. The activity of natural materials is increased by acid hydrolysis. 5. Active dried cell preparations catalyzing the breakdown of formate to CO_2 and H_2 may be obtained by vacuum drying.

TABLE III. Stimulation of FHL Activity of Vacuum-Dried Preparations of *Aerobacter aerogenes*.

No additions	FHL activity* yeast extract (1-2 mg)	Hydrolyzed† yeast extract (1-2 mg)
170	340	840
1700	1900	2800
300	380	860
360	420	1000
1100	1300	2240
62	106	164
910	940	1400

* Expressed as $Q_{(H_2)}$; see Table II.

† 121°C, .9 N H_2SO_4, 3 hr.

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Effect of Steroids upon Resistance of Skin to Intracutaneous Injection. (19362)

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Cortisone inhibits the spreading action of hyaluronidase in the skin(1,2). The physical properties of the ground substance of connective tissue are probably related to the metabolism of hyaluronic acid, and evidence has been presented that this is partly under control of the adrenocortical steroids(3,4). Atrophic changes in the skin and inhibition of hair growth have also been described in animals treated with cortisone(5,6).

The present investigation demonstrates the effect of hyaluronidase and of cortisone upon the resistance offered by the skin to the intracutaneous introduction of fluids, as measured by the amount of pressure required to perform an intracutaneous injection under carefully controlled conditions.

Material and methods. Young adult male Holtzman rats of about 200-250 g body weight were used. Observations were made under light ether anesthesia. Injections were made in comparable shaved areas of the skin of the back in all animals. The apparatus used is illustrated in Fig. 1. The syringes and Tygon tubing connections shown in stippling were filled with water. The solution to be injected was drawn into syringe B, and needle A was inserted into the skin, care being taken not to get the point of the needle into the subcutaneous tissue. The automatic infusion pump G was then started, and injection continued until 0.05 ml of fluid was delivered from syringe B. The speed of the infusion pump was set so that the plunger of syringe D moved at a rate of 22 mm per minute. When care was taken to keep the volume of injected fluid and the rate of injection constant, the pressure readings (obtained on drum F) were remarkably uniform among different animals treated in the same way on the same day. Variations in volume or speed of injection markedly affected the pressure readings obtained. The solution used in syringe B was either physiological saline or 0.33% testicular hyaluronidase (a commercial preparation purchased from The Treemond Co., New York) in saline. The pressure readings reported were obtained by subtracting the pressure required to extrude the fluid from the needle (*i.e.*, in air) from that obtained when the needle was in the skin. From 4 such determinations on each animal, the mean for each

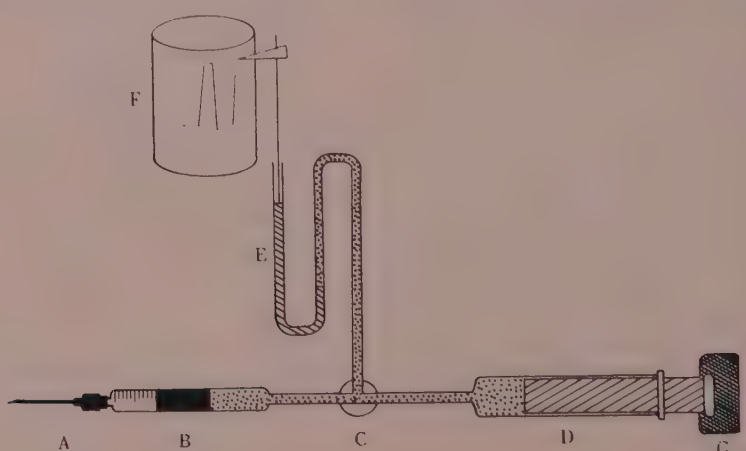

FIG. 1. Apparatus used for measuring intracutaneous pressure. A. 27 gauge ½-inch needle. B. Tuberculin 1 cc syringe with small segment of plunger (shown in black). C. 3-way stopcock. D. 5 cc syringe. E. Mercury manometer. F. Kymograph. G. Constant rate infusion pump.

TABLE I. Pressure in mm of Mercury Required to Introduce Fixed Volume of Fluid Under Controlled Conditions Intracutaneously in Rats.

Steroid	Inj. fluid	Day 1	2	3	4	Mean
None	S*	75.75	75.75	68.25	60	68.7
	H	56.25	37.25	49	38.25	45.2
Testosterone	S	82.50	70	66.25	49.25	67
	H	48.75	40.25	48	42.25	44.8
Desoxycorticosterone	S	75.50	75.25	64.75	53.75	67.3
	H	63.25	43	39	41.50	46.7
Compound S	S	61	69	58	58	61.5
	H	50.75	40.75	43.50	44.25	44.8
21-acetoxypregnenolone	S	78.50	71	62	58.50	67.5
	H	52.25	53.25	50.75	42.50	49.7
Cortisone	S	116	106.25	94	81	99.3
	H	84	63.25	70	67.25	71.1

Analysis of variance

Source of variation	df	F
(1) Days	3	3.68
(2) Hyaluronidase	1	32.71 s
(3) Error, hyaluronidase	3	1
(4) Steroids	5	50.42 v
(5) Error, steroids†	83	1
Total	95	

* S = Saline. H = Hyaluronidase.
† Including non-significant interactions.
Standard error for a single hyaluronidase observation = ± 18.4 mm Hg.
" " " " " steroid " = ± 6.72
s = significant ($.01 < P < .05$). v = very highly significant ($P < .001$).

group was calculated. Steroids were administered intramuscularly in saline suspension, 0.5 mg daily for 4 days, and the pressure measurements were made 2 hours after the last injection. The steroids used were cortisone acetate (Cortone Merck), desoxycorticosterone acetate (D.C.A.), 17 hydroxy-11-desoxycorticosterone acetate (Reichstein's Compound S), 21-acetoxy-pregnenolone (artisone), and testosterone propionate.

Experimental. Preliminary experiments demonstrated that cortisone alone of the steroids tested markedly increased the resistance of the skin to intracutaneous injection, that this effect was observed whether hyaluronidase was present in the injection fluid or not, but that the picture was complicated by the presence of considerable day-to-day variation. Therefore, an experiment was conducted according to an experimental design as detailed by Cochran and Cox(7), by means of which the effects of the various treatments of the animals could be assessed against the background of daily variation. The design of the experiment is clear from an inspection of Table I; each individual figure in the table is the average of 2 animals, so that a total of 96 animals was used, 16 on each steroid, 8 with hyaluronidase, and 8 without hyaluronidase in the fluid injected intracutaneously.

The data clearly demonstrate the following: 1) The amount of pressure required to make an intracutaneous injection under the conditions of these experiments, with saline as the injection fluid, varies from about 50 to 80 mm Hg, with an average of about 67 mm Hg. 2) If hyaluronidase is present in the injection fluid, the injection pressure is reduced by 21.5 mm Hg on the average, or about one-third. This finding is consistent with results of others(8,9). 3) Neither testosterone, D.C.A., Compound S, nor artisone has any effect upon the injection pressure, either in the presence or absence of hyaluronidase. 4) Cortisone increases the injection pressure by about 20 mm Hg, either in the presence or absence of hyaluronidase. The statistical

analysis shows no significant interaction between steroid treatment and hyaluronidase.* Therefore, the effect of the cortisone is independent of the effects of hyaluronidase.

Discussion. It is evident that cortisone has a highly specific effect in increasing the resistance offered by the skin to intracutaneous injection. Even though hyaluronidase lowered the injection pressure, and cortisone increased it, it is clear that this effect of cortisone is not due to its antagonism to hyaluronidase. This is evidenced by the fact that the cortisone had an equally great effect when hyaluronidase was not included in the injection fluid, and, as mentioned above, the independence between effects of steroid and effects of hyaluronidase. Furthermore, in previous experiments in this laboratory, some of which are published(2), it had been found that all the steroids used, with the exception of D.C.A. produced, when administered to intact rats, marked inhibition of hyaluronidase as measured by the spreading of intradermal fluids.

It must, therefore, be assumed that cortisone treatment produced some change within the tissue of the skin, presumably in the ground substance of the connective tissue, to account for the observed resistance of the tissue to introduction of fluid. Previous investigators have emphasized the influence of cortisone upon permeability(4,10) and probably the effect herein described is in some way related to that described by these authors, but the relative effects of the various steroids herein described are very different from those observed by Seifter and coworkers(10,11), who found D.C.A. to have an action opposite to that of cortisone, and artisone to be as effective as cortisone. The action of cortisone seen in our experiments is a more specific one than those observed by the authors cited; that is, a number of compounds which had an action similar to that of cortisone in Seifter's experiments were inactive in the present experiments. The collagen and amorphous ground substance of the skin are diminished and the synthesis of chondroitin sulfate is inhibited by cortisone(12). These metabolic changes may produce alterations in the physical properties of the skin which might account for the effects observed. Such alterations might include a change in the hydrophilic properties of the colloids in the skin so that aqueous fluids are less readily absorbed.

Summary. 1. Measurements have been made of the resistance offered by the skin to the intracutaneous introduction of fluid, as determined by the amount of pressure required to perform an intracutaneous injection under carefully controlled conditions in adult male rats. 2. Systemic administration of cortisone markedly increased the resistance of the skin to injection. Hyaluronidase, incorporated in the fluid administered intracutaneously, lowered the injection pressure. The effect of cortisone, however, was shown to be independent of the effect of the steroid upon hyaluronidase. Testosterone, desoxycorticosterone, compound S, and 21-acetoxypregnenolone were without effect under the conditions of these experiments.

* We are indebted to Mr. Gordon Thomas of the Merck Institute for Therapeutic Research for statistical analysis of the data, and assistance in the experimental design.

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Changes in Electrolyte and Water Composition after Glucose in Unprotected and Epinephrine-Protected Rats. (19363)

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Several investigators(1-3) have reported that the level of plasma potassium was definitely lower than the initial K level about 20 minutes after injection of epinephrine or insulin in man and other animals. Recently, Dury, *et al.*(4) reported that the pattern of change in plasma potassium concentration and the extent of fall from initial level, in the course of 2 hours after epinephrine, was different in patients with Addison's disease and with idiopathic epilepsy than the response in normal humans similarly tested. Tum Suden(5) reported that after sympathetic blockade with ergotamine, in rats, the toxic dose of potassium was diminished; and Dury and Johnston(6), showing that there was a significant decrease in plasma and skeletal muscle concentration of potassium 60 minutes after an injection of epinephrine, suggested that epinephrine may have a regulatory role in the potassium balance of the organism.

In a report from this laboratory(7) it was shown that pretreating adrenalectomized rats with a single physiological dose of epinephrine 60 minutes before admitting 2 ml of a 50% glucose solution into their stomachs protected about 80% of these animals from a fatal hyperkalemia and cardiac changes. Non-pretreated adrenalectomized rats developed a hyperkalemia and cardiac changes leading to a high percentage of mortality within a short time after glucose was given. The source of the rapidly mobilized plasma potassium in the unprotected rats, and the manner in which epinephrine-pretreatment had "blocked" the onset of hyperkalemia was not demonstrated. It is the purpose of this paper to present the changes in tissue electrolyte and water concentration in unprotected and epinephrine-protected rats after a glucose load.

Materials and methods. Male Wistar rats weighing 225-250 g were used either 6-7 days or 12-13 days after adrenalectomy. The animals were maintained on 1% NaCl drinking solution and were given a chow biscuit *ad libitum* and greens twice weekly. After an overnight fast the animals were anesthetized with n-methylcyclohexenyl-methyl barbituric acid (EVIPAL). The rats were then either given 2 ml of a 50% glucose solution (*c.p.* Dextrose, Merck) directly into the stomach by means of a fine plastic tube, or first pretreated with epinephrine 60 minutes before the glucose solution was administered. The epinephrine (Adrenaline tablets, Parke, Davis and Co.) was prepared fresh in 0.9% NaCl and injected subcutaneously at a dose level of 0.04 mg/100 g body weight (approx. 0.5 ml). After the glucose solution had been admitted into the stomach each of the rats was observed carefully for the first signs of respiratory difficulty. When this was observed blood was withdrawn from a cardiac puncture into a dry syringe containing 1 drop of heparin (Liqeamin, Organon, 10 mg/ml). On the basis of previous evidence(7) those rats which showed no signs of failure for 20 minutes after the glucose load were accepted as probably surviving the procedure and blood was obtained from these shortly thereafter. The control groups for this study were rats 6 and 12 days after adrenalectomy which had not been treated in any way, and a group of rats 6 days after adrenalectomy which were killed 60 minutes after the epinephrine injection and tissues obtained for analyses of electrolyte and water composition. The procedures followed for analyses of electrolyte and water composition of blood and tissues was the same for all animals and has been described in detail in a previous paper(6). Tissue chloride content was determined by the method of Van Slyke(8), and plasma chloride by the method of Schales and Schales(9). Plasma glucose was determined by the photometric method of Kingsley & Reinhold(10). Tissue and plasma sodium and potassium content was determined with the aid of an internal lithium standard flame photometer.

Results. The present investigation was

TABLE I. Water and Electrolyte Composition of Plasma of Non-Pretreated and Epinephrine-Pretreated Adrenalectomized Rats After a Glucose Load per os.

Animals used	Survival time after glucose, min	% plasma water	m.eq./kg plasma water Cl	K	Na	Plasma glucose, mg %
Rats 6-7 days after adrenalectomy						
a. No inj. (9)		94.1 ± .17†	112.8 ± 1.3	5.28 ± .19	147.7 ± 2.2	45
b. " " + glucose* (9)	<5 (5)	93.8 ± .17	*105.8 ± 2.6*	*7.03 ± .31*	149.6 ± 3.3	54
	10–15 (2)	94.4	110	5.88	147.6	72
	>20 (2)	93.8	106.7	4.62	152.1	118
c. Epinephrine‡ (13)		93.7 ± .11	110.8 ± 1.1	4.31 ± .4	150.8 ± 1.7	159
d. " + glucose§ (9)	10 (1)	93	115.2	5.57	×	×
	16–19 (3)	92.8	113	5.60	×	218
	>20 (5)	93.2 ± .16	115.1 ± 3.3	4.47 ± .56	152.3 ± 1.8	239
Rats 12-13 days after adrenalectomy						
e. No inj. (6)		94.2 ± .1	105.8 ± 2.5	5.79 ± .26	147.9 ± 2.1	50
f. " " + glucose (6)	<5 (5)	94 ± .19	103.1 ± 1.9	*6.68 ± .41*	147.5 ± 2.1	94
	>20 (1)	94	99.2	4.52	147.4	120
g. Epinephrine + glucose (5)	>20 (4)	93.5 ± .2	102.7 ± 1.3	*3.97 ± .08*	144.1 ± .8	239

* 2 ml-50% glucose sol. into stomachs.
† Mean ± S. E.
‡ Cardiac blood and tissues taken 60 min after epinephrine pretreatment at .04 mg/100 g wt.
§ 2 ml-50% glucose sol. into stomach 60 min after epinephrine pretreatment at .04 mg/100 g wt.
Figures in italics are significantly different from controls.

designed to detect in the non-pretreated and epinephrine-pretreated groups: 1) the changes, if any, from the respective controls of the composition of water and electrolytes in the plasma, skeletal muscle and liver of rats which showed impending mortal failure after the glucose load was placed in their stomachs; and 2) the changes, if any, in the plasma, muscle and liver composition of rats which obviously were surviving the administration of the glucose load.

Survival. The number of rats in the non-pretreated and epinephrine-pretreated groups which showed signs of failure at several time-periods after glucose *per os* and those surviving the procedure are shown in the second column of Table I. It is apparent that epinephrine-pretreatment had protected a large percent of the rats from failure after the administration of glucose. Similar survival and mortality results have been reported from this laboratory(7) for rats 16 days after adrenalectomy and similarly treated.

Plasma changes. The data presented in Table I show that impending mortal failure in rats 6 and 12 days after adrenalectomy was related to an increased plasma potassium concentration following the admission of a glucose load into their stomachs. In the non-pretreated series (b,f) there was a marked increase in the potassium concentration in the groups which showed signs of failure within 5 minutes after glucose. In the three rats of this series which showed no signs of failure throughout the designated survival period the plasma K concentration was found to be in the range of normal intact controls and somewhat lower than the mean value of the adrenalectomized controls. The relationship between increased plasma potassium concentration and low survival rate demonstrated in the non-pretreated series (b,f) is supported by the data of the epinephrine-pretreated series (d,g) in which a large percentage of the rats survived the glucose load. It is evident that a rapidly developed hyperkalemia was the single plasma change which followed the administration of the glucose load in a large number of rats in the non-pretreated series. Epinephrine-pretreatment, however, had somehow altered conditions so that after the glucose load the plasma potassium concentration had been maintained within normal range in this series of rats.

Muscle and liver changes. Non-pretreated series. The muscle electrolyte and water composition of the groups of rats in series b and f after the glucose load was found to be not

TABLE II. Tissue Water and Electrolyte Composition of Non-Pretreated and Epinephrine-Pretreated Adrenalectomized Rats After Glucose.

Series	Survival time, min*	Muscle water, g/kg wet tissue			Muscle electrolytes, m.eq./kg wet tissue			Total water, g/kg wet liver	Liver electrolytes, g/kg wet tissue	
		Total	ECW	ICW	Cl	K	Na		K	Na
					Rats 6-7 days after adrenalectomy					
a.†	(controls)	770 ± 1‡	117 ± 4	653 ± 5	13.7 ± .6	90.1 ± 1.6	36.8 ± 1.9	719 ± 4	77.3 ± 3.3	40.5 ± 3
b.	<5 (5)	770 ± 4	107 ± 3	663 ± 5	12 ± .4	86.3 ± .7	33 ± 3	722 ± 5	68.9 ± .6	42.8 ± 2
	10–15 (2)	772	125	656	13.4	93.6	30.1	731	79.3	37.3
	>20 (2)	766	115	651	12.9	91.7	34.9	721	74.7	42.5
c.	(controls)	775 ± 1	129 ± 4	646 ± 3	14.9 ± .4	85 ± 1.1	40.8 ± .8	720 ± 2	83.9 ± 2.2	37.1 ± 2
d.	10 (1)	764	105	659	12.7	92	38.2	709	82.4	43.1
	16–19 (3)	759	114	645	13.2	91.2	35.4	724	84.6	35.4
	>20 (5)	764 ± 1	127 ± 2	637 ± 1	14.6 ± .5	93.5 ± 3.1	33 ± 3.2	714 ± 4	81.7 ± .8	38.6 ± 2
					Rats 12-13 days after adrenalectomy					
e.	(controls)	768 ± 7	112 ± 2	656 ± 8	12.5 ± .4	85.1 ± 1.3	38.4 ± 1.8	721 ± 2	81.6 ± 2.5	32.7 ± 2
f.	<5 (5)	775 ± 3	114 ± 5	661 ± 5	12.3 ± .7	87.5 ± .9	38.9 ± 1.8	729 ± 3	77.9 ± 2.2	40.7 ± 2
	>20 (1)	775	111	664	11.6	97.8	33.4	733	81.6	34.9
g.	>20 (4)	777 ± 2	131 ± 8	646 ± 6	14.1 ± .7	88.3 ± 2.7	35.3 ± 3.3	723 ± 3	80.2 ± 4.6	37.6 ± 4

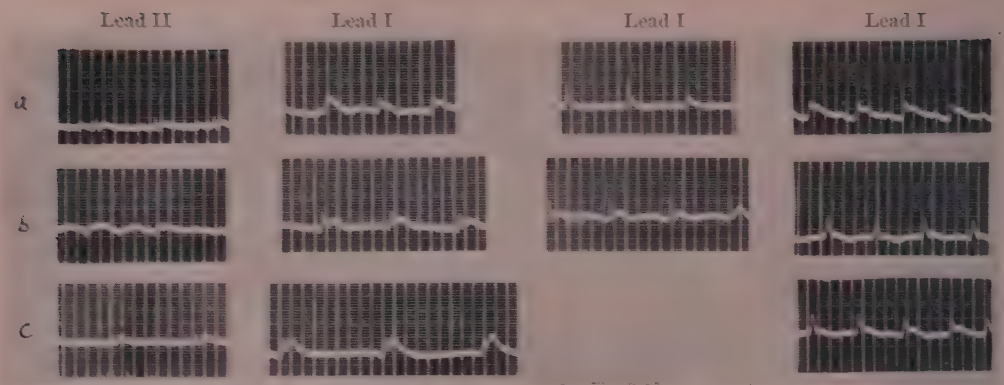
* Tissues taken at indicated survival times for analyses. † See Table I for legends. ‡ Mean ± S. E.
No. of animals in group in parentheses. Figures in italics are significantly different from controls.

different from the mean control values (Table II). However, the results of analyses of liver composition show a marked decrease in the liver potassium concentration in the groups of this series which failed within 5 minutes after the glucose load. This change, however, was not found in the few rats of this series which had survived the glucose administration for 10 minutes or more. Evidently a rapid "unloading" of liver potassium had taken place in a large number of the rats not pretreated with epinephrine.

Muscle and liver changes. Epinephrine-pretreated series. The results of analyses of muscle electrolyte and water composition of the groups in series d and g show that only the potassium concentration was significantly increased after the glucose load *per os* (Table II). This change occurred not only in the rats which had shown no signs of failure, but was found in the few rats of this series which failed 10-19 minutes after the glucose load. The results of analyses of liver composition in this series revealed that neither the concentration of liver potassium nor the other constituents had been altered after the glucose load *per os*. The finding is in marked contrast to the large decrease in liver potassium concentration found in the non-surviving groups of the non-pretreated series.

In summary, the results of the analyses of muscle and liver composition in these series of rats showed that in the non-pretreated series the essential finding in those groups of rats presenting signs of mortal failure within 5 minutes after glucose *per os* was a marked decrease in liver potassium concentration whereas muscle K concentration remained unchanged. These results were different from those found in the epinephrine-pretreated series of rats in which liver K concentration was found unchanged after the glucose load from the mean control value and the muscle potassium concentration was found to be increased.

Discussion. This investigation was undertaken in an attempt to gather evidence which would elucidate the basis for the difference in the effect of a glucose load *per os* in non-pretreated and epinephrine-pretreated adrenalectomized rats. The data in Table I showed



Rat #2G. Non-pretreated. a. Before glucose *per os*. b. 1½ min after glucose. c. 3 min after glucose; signs of failure; before blood was drawn.

Rat #12G. Non-pretreated. a. Before glucose *per os*. b. 3 min after glucose. c. 5 min after glucose; signs of failure; before blood was drawn.

Rat # 3G. Non-pretreated. a. Before glucose *per os*. b. 17 min after glucose; signs of failure; just before cardiac blood was drawn.

Rat #8G. Epinephrine pretreated. a. 60 min after epinephrine and just before glucose. b. 5 min after glucose. c. 21 min after glucose; no signs of failure; just before cardiac blood drawn.

FIG. 1. Electrocardiographic records of non-pretreated and epinephrine-pretreated adrenalectomized rats before and after glucose *per os*.

that epinephrine-pretreatment protected rats 6 and 12 days after adrenalectomy from a high mortality rate which otherwise ensued shortly after a glucose load was placed into the stomachs of the non-pretreated adrenalectomized rats. The results indicated that rapid increase in plasma potassium concentration, the marked decrease in liver potassium composition, and the low survival rate found in the non-pretreated rats shortly after a glucose load were apparently related. It is suggested that the sequence of events after the glucose load was probably as follows: a rapid release of liver potassium into the blood stream followed by a marked increase in the level of blood potassium. This rapid change affected the electrical activity of the heart which was evidenced by cardiac failure and standstill found in this series of rats. Reproductions of the electrocardiographs of some of the non-pretreated and epinephrine-pretreated rats before and after the glucose load are shown in Fig. 1. The altered cardiac activity in the former group are clearly consonant with the findings of increased potassium concentration and support the explanation for the rapid failure of a large percentage of rats in this series.*

Insofar as the possible manner in which epinephrine-pretreatment protected the adrenalectomized rats after a glucose load, the available data does not warrant more than speculation of the mechanism. However, two facts are clear from the results presented: 1) The level of plasma potassium was maintained in this series after the glucose *per os* at approximately the control level in a large percentage of the animals. Even in the few rats of this series which failed during the allotted survival period there was not the sharp increase in potassium level found in the rats of the non-pretreated series. 2) The results of analyses of the liver composition in this series suggest that a "release" of liver potassium did not occur after the glucose load. It had been shown in a previous study(6) that the concentration of potassium in plasma and muscle was significantly decreased from its initial level 60 minutes after an epinephrine

* EKG changes will be reported later. Rats were attached to a Cambridge Electrocardiograph with German Silver electrodes, designed by Drs. Hundley and Pecora of the National Institutes of Health, and made by Surveyor Service Co., Silver Springs, Md. Electrocardiographic paper was fed through machine at a speed of 100 mm/sec.

injection. This status before the administration of the glucose load could explain the evident maintenance of the plasma level in these rats. Consideration, also, should be given to the possibility that the epinephrine-pretreatment had resulted in metabolic adjustments related to increased liver glycogenolysis and peripheral utilization of glucose in these animals so that the sudden imposition of a glucose load *per os* could be taken into the metabolic pool without seriously impairing the functions of the animal.

Summary. 1. The survival rate, and the results of tissue composition are presented for rats showing signs of failure and those surviving the administration of a glucose load *per os* (2 ml– 50% solution) 60 minutes after epinephrine-pretreatment (0.04 mg/100 g wt) and those which were not pretreated. 2. Signs of mortal failure in a large percentage of the non-pretreated series of rats shortly after glucose *per os* was associated with a significant increase in the plasma potassium concentration and a significant decrease in the liver potassium composition only. 3. In the epinephrine-pretreated series a large percentage of the rats survived the glucose load *per os*. It was shown that the level of plasma potassium and the liver potassium concentration were unchanged from the control values. The muscle potassium concentration was found significantly increased after glucose in this series. 4. Representative EKG records of non-protected and epinephrine-protected rats are shown. The EKG changes in the former group are consonant with the analytical results indicating that hyperkalemia was responsible for failure of these animals. 5. The results were discussed as suggestive of: a) the sequence of changes in the non-pretreated series which led to mortal failure and low survival rate shortly after the glucose *per os*; and b) the possible manner in which epinephrine-pretreatment had altered the metabolic status of these rats before the glucose *per os* was given and thereby "protected" them from the changes in tissue composition found in the former group.

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Specific Inhibition of Precipitation as an Aid in Antigen Analysis with Gel Diffusion Method.* (19364)

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Antigenic material derived from bacteria, animal tissues, and similar sources is usually immunologically extremely heterogenous. Even chemically pure synthetic antigens almost always exhibit a complex immunological pattern giving rise to a number of distinct antibodies. The heterogeneity of antigens is not always demonstrable in the ordinary flocculation, agglutination or complement fixation procedures. More information can be gained by the use of the quantitative precipitation reactions performed according to the princi-

* This study was aided by a grant from Karolinska Institutet, Stockholm.

FIG. 1. Antigen analysis according to Ouchterlony. In the agar, poured in a Petri dish, three basins are spared. On the picture one sharp line of precipitate can be seen giving reaction of identity.

ples laid down by Heidelberger, but this method is complicated, requires comparatively large amounts of antigen and immune serum, and is not always sufficiently sensitive. The gel precipitation method as developed by Ouchterlony presents some advantages: very high resolving power, as practically any number of components can be individually demonstrated, and sensitivity of the same order as any of the other serological methods. This method is based upon the observation that antigens and their corresponding antibodies, diffusing towards each other in a gel, react by forming sharply defined lines of precipitation, the sites of which are dependent upon the diffusion rates and the concentrations of the reactants. By the introduction of 3 diffusion centers, 2 different antigen or antibody systems can be directly compared by means of phenomena of interaction indicating immunologic identity, partial identity or non-identity of individual components (Fig. 1-4). When working with preparations containing numerous antigenic components, it may be difficult to analyze and identify the individual components. Fusion of the lines and the very multiplicity of precipitates may give rise to analytical difficulties which can be hard to overcome. For certain analytical purposes, there is thus a need for a modification, which makes it possible to study in detail one or some of the components. The present paper reports some experiments concerning the principle and possibilities of such a modification.

Materials. Antigens. Diphtheria toxin produced on the medium of Philippe and Loiseau, dialyzed and concentrated by ultrafiltration. Tetanus toxin produced on the broth of Martin, passed through a Seitz filter and concentrated by ultrafiltration. Normal rabbit

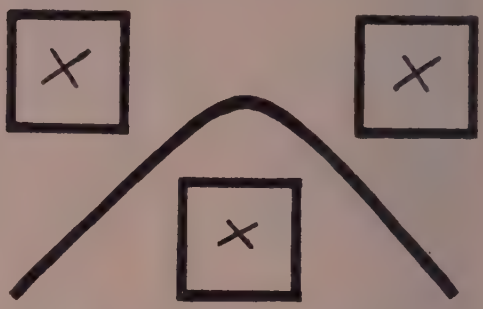

FIG. 2. Reaction of identity: If 2 antigenic preparations, both containing the antigen X, diffuse from the top basins, they will form a line of precipitate with the anti-X-serum diffusing from the bottom basin.

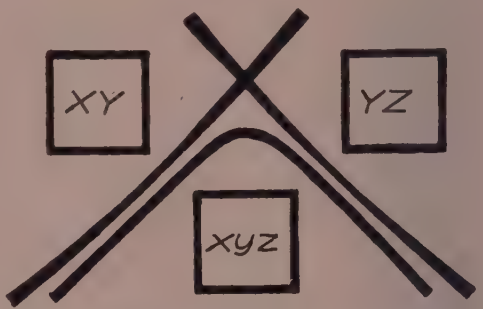

FIG. 3. Reaction of non-identity: If 2 antigenic preparations, both containing an antigenic component not in common with the other, diffuse from the top basins each one will form a line of precipitate with the corresponding antibody from the bottom basin. The lines do not interfere with each other. Further, common components give rise to a reaction of identity.

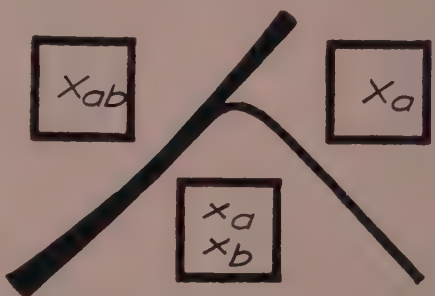

FIG. 4. Reaction of partial identity: If one of the antigens has 2 antigenic properties, one of which is in common with the other antigen, a partial fusion of the precipitates will occur.

plasma from healthy animals. Bone-marrow extract: bone-marrow from rabbits was extracted with ether, lyophilized, ground in a ball mill, and again extracted with ether. The dry powder, obtained after removal of the ether, was extracted with saline, and centrifuged. The clear supernatant was used as antigen(1).

Hyperimmune sera. Diphtheria antitoxin from a horse immunized with crude toxoid. Tetanus antitoxin from a horse immunized with toxoid. Anti-bone-marrow serum from a horse immunized with a total amount of 32.75 g dry powder of rabbit bone-marrow during a period of 4 months. (For further information see the same reference as given above).

Diffusion medium. Good quality agar was dissolved in 30 parts of distilled water, precipitated when hot with 0.5% calcium chloride, filtered, and left to congeal. It was then cut into small pieces, and rinsed in running tap water for 72 hours. After melting, an equal amount of 1.6% saline was added, and further merthiolate and methyl orange to final concentrations of 1:10,000 and 3:100,000, respectively. This agar solution was poured in Petri dishes to form a thin layer. After congelation a second layer was established, in which 3 basins were spared with the aid of a metal matrix (Fig. 1).

Methods. All diffusion plates were handled at 20°C. In order to limit evaporation of water from the gel, the plates were kept under glass cover. Sterile conditions were maintained. All plates were made in duplicate. If the precipitation patterns were not identical, both plates were discarded. For immunoprecipitations, concentration gradients were established in the diffusion medium by filling antigen preparations in the two lateral basins and immune serum in the bottom one. The basins were filled once daily for 3 consecutive days. Specific inhibition of precipitation was achieved by introduction of antigen or antibody into the gel before the precipitation experiment was started. For this purpose all 3 basins were filled for 3 consecutive days with the preparation, which was then allowed to diffuse completely into the gel. Precipitation was recorded photographically after 10 days. The plates were illuminated obliquely by reflected short wave light from a mercury lamp. Through the Tyndall effect the precipitates stood out distinctly against the orange background and pictures of high contrast were obtained with blue-sensitive film.

Experimental. In varying concentrations, the following antigens were tested for their suitability for the present experiments: diphtheria toxin, tetanus toxin, and staphylotoxin. These preparations gave distinct lines of precipitation with their corresponding antisera. As the precipitation patterns of the diphtheria and tetanus systems were the most typical and distinct ones, easy to reproduce, and could be produced simultaneously on the same side in the same plate, they were considered the most suitable ones for this study. In diffusion plates the bottom basins were filled with a mixture of diphtheria and tetanus antitoxins, the top left basins with diphtheria toxin, and the top right ones with tetanus toxin. Two distinct groups of precipitates appeared, showing the pattern of a "non-identity" reaction: on the left side that of the diphtheria system, and on the right side that of the tetanus system (Fig. 5). Although the precipitates of both preparations were complex, each pattern presented such a typical configuration that it was readily recognizable. Next, the diffusion plates were prepared beforehand with diphtheria antitoxin of a certain concentration, as described under methods, following which precipitation was attempted with the same reactants as in the previous experiment. As demonstrated in Fig. 6, the diphtheria precipitates in this case appeared closer to the top

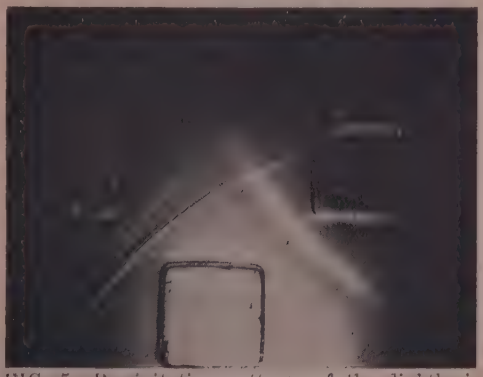

FIG. 5. Precipitation patterns of the diphtheria (left) and the tetanus (right) systems.

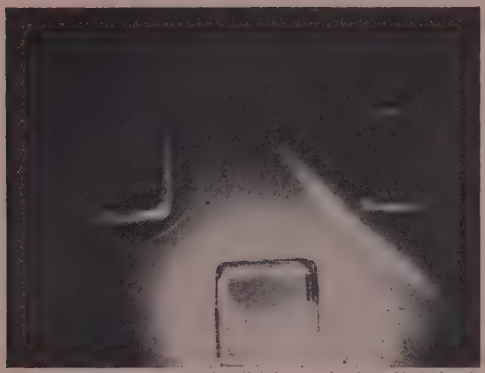

FIG. 6. Incomplete inhibition of the diphtheria lines (left).

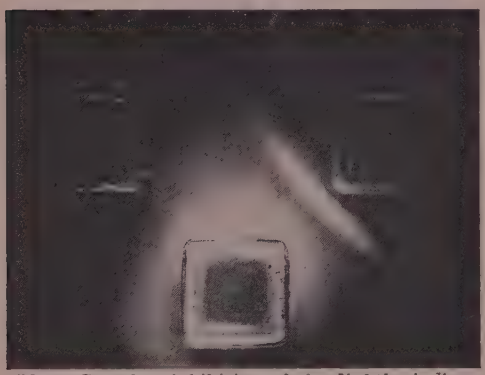

FIG. 7. Complete inhibition of the diphtheria lines.

left basin. The tetanus lines were not influenced. If in preparation of the plates a higher concentration of diphtheria antitoxin was employed the diphtheria lines could be completely inhibited (Fig. 7).

In principally the same way a systematic study of the inhibiting effect of each one of the 4 components of the present antigen-antibody system was carried out. The results are summarized in Table I. In each case the preparation of the medium with either one of a pair of reactants, antigen or antibody, in a sufficient concentration resulted in a complete inhibition of all precipitates belonging to that particular reaction, whereas the site and appearance of the precipitates of the non-inhibited reaction were not influenced. The results so far obtained indicate that the method might be useful in the antigen analysis of highly complex biological materials. This possibility was tested on the immunoprecipitation of rabbit bone-marrow and anti-bone-marrow hyper-immune horse serum.

Preliminary experiments had shown that this system presented a very complex precipitation "spectrum" with Ouchterlony's method. Furthermore, with rabbit plasma as antigen the same hyper-immune serum produced a number of precipitate lines (Fig. 8). Although some "reactions of identity" between the two antigens were obvious, a thorough analysis of the complicated pattern was not feasible.

During the preparation of the bone-marrow antigen, small amounts of blood are unavoidably retained in the material. The appearance in the hyper-immune serum of antibodies to plasma constituents is thus readily explained. It is reasonable to presume, however, that the bone-marrow contains additional cellular antigens that are not demonstrable in normal plasma. By addition of plasma to the diffusion medium in concentrations sufficient to suppress the appearance of lines belonging to the plasma-antiserum system it should be possible to bring out and analyze the reactions of the non-humoral, precipitate-forming components of the bone-marrow. Accordingly, diffusion plates were pretreated with rabbit plasma. After completed diffusion, precipitation tests were set up with anti-bone-marrow serum in the bottom basins, a preparation of rabbit bone-marrow in the top left basins, and rab-

TABLE I. Inhibition of Appearance of Precipitation. Arrangements and results.

Inhibitors	Diphtheria serum	Tetanus serum	Mixed sera	Antigens, toxins
	D		D	Diphtheria
	D	T	D+T	Mixed
		T	T	Tetanus
Diphtheria toxin				Diphtheria
		T	T	Mixed
		T	T	Tetanus
Tetanus toxin	D		D	Diphtheria
	D		D	Mixed
				Tetanus
Diphtheria serum				Diphtheria
		T	T	Mixed
		T	T	Tetanus
Tetanus serum	D		D	Diphtheria
	D		D	Mixed
				Tetanus

D = Appearance of precipitation pattern of the diphtheria system.

T = Appearance of precipitation pattern of the tetanus system.

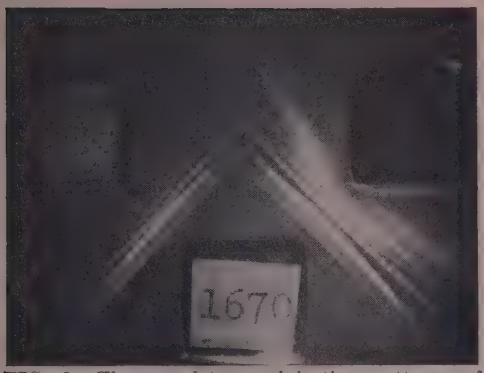

FIG. 8. The complete precipitation patterns of bone-marrow (left) and plasma (right).

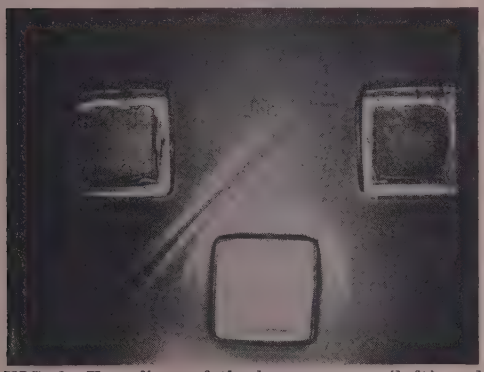

FIG. 9. Four lines of the bone-marrow (left) and incomplete inhibition of the plasma lines.

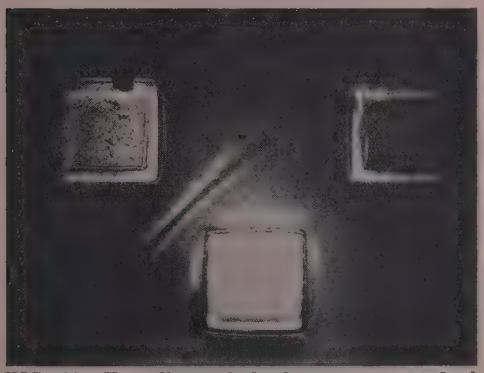

FIG. 10. Four lines of the bone-marrow and almost complete inhibition of the plasma lines.

bit plasma in the top right basins. As controls untreated plates were also used.

The appearance of lines corresponding to plasma antigens was not completely inhibited as shown by concentric precipitates around the antibody basins. However, the development of 4 distinct precipitates was evident on the left side, whereas no corresponding lines were seen on the other side (Fig. 9), a fact indicating that the bone-marrow preparation contained antigens not present in normal plasma.

It was possible by increasing the concentration of rabbit plasma in the medium to suppress almost completely the formation of plasma lines without any visible effect upon the 4 precipitates characteristic of the bone-marrow preparation (Fig. 10).

Discussion. Ouchterlony (2-8) showed that the site of an immunoprecipitate in the diffusion medium was determined i.a. by the ratio of concentrations of antigen and antibody in the diffusion centers. By variation of the concentration ratio the precipitate was displaced towards the basin with the lower relative concentration. Theoretically it should be possible to establish such conditions that the precipitate will appear not in the gel, but virtually within the basin with the lower concentration.

Introduction of one reactant in the medium prior to addition of the other is no doubt the best method to secure the high concentrations necessary to retain the precipitate within the boundaries of the basin. In this way a barrier is formed in the very wall of the basin preventing diffusion of the reactant into the medium. In principle this is equivalent to specific absorption of a serum, provided that the diffusion of other reactive constituents is not influenced by the formation of specific precipitates in the wall of the basin. If the latter condition is fulfilled the procedure would provide a simple means of eliminating selectively certain lines from a complicated precipitation spectrum, thus facilitating an antigen analysis.

From Ouchterlony's studies it would appear that the formation of an immunoprecipitate does not prevent the diffusion of constituents not involved in the specific reaction. However, before application of the procedure outlined in this paper to any actual problem it was deemed necessary to study the effect on artificial mixtures of antigens and antibodies, respectively. The system chosen for this study contained diphtheria and tetanus toxins and their antitoxins. The individual precipitation patterns of each toxin was found to

be so characteristic that identification was possible beyond doubt. The results showed clearly that elimination of the lines belonging to one component by pretreatment of the medium with either antigen or antibody had no influence whatever upon the formation of the precipitate characteristic of the other. It would seem, therefore, that the procedure can be safely applied in the analysis of complex antigenic systems.

Preliminary experiments along these lines with antigens prepared from rabbit bone-marrow gave promising results. In a subsequent paper the application of the procedure in the analysis of the antigenic structure of different organs will be described(9).

Summary. A modification of Ouchterlony's gel diffusion method for antigen analysis is described. It is based on the following observations: 1. By pretreatment of the diffusion medium with sufficient amounts of one component of a complex immunologic system the subsequent appearance in the medium of precipitates corresponding to this particular component could be completely inhibited. 2. The diffusion of the other components of the same system and the formation of other immunoprecipitates was not influenced. 3. By incorporation in the diffusion medium of individual components of a complex immunologic system it is thus possible, by elimination of some of the lines, to simplify the precipitation spectrum and facilitate the antigen analysis.

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Serological Analysis of Components in Hemopoietic Tissue.* (19365)

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In a previous paper(1) by Björklund and Hellström the biological effects of a horse hyperimmune serum against rabbit bone-marrow were described. Rabbits, after receiving intravenous injections of this serum, displayed symptoms referable on one hand to a histopathologically demonstrable aplasia of the hemopoietic system and degeneration of the circulating blood cells, on the other hand to a general toxic effect. Administration of large serum doses invariably caused the death of the animals. It seemed probable that antibodies were primarily responsible for the effects observed.

It might be assumed that the preparations of bone-marrow used for immunization of the horses contained a number of substances with distinct immunological properties. Among these, some are presumably specific for different elements of the hemopoietic system, while others originate in capillary endothelium, connective tissue, serum and similar sources not intrinsically connected with the hemopoietic apparatus proper. By means of serological *in vitro* studies it should be possible to differentiate between the antigenic components and examine their distribution in different organs and tissues. The same methods might also be applied as a guide in attempts at isolation and purification of the biologically interesting antigens. The present paper reports

* This study was aided by a grant from Karolinska Institutet, Stockholm.

some preliminary experiments along these lines.

Materials and methods. Antigens. Red bone-marrow, liver, spleen, lung and adrenals were obtained from rabbits used for pregnancy tests according to Friedmann-Schneider. The organs were removed within 2 hours after the animals had been sacrificed and immediately frozen at −20°C. Batches of tissue of each kind were ground and extracted 3 times with ether at +5°C. After removal of the ether, the residue was lyophilized, ground in a ball mill, and again extracted with ether. One gram of the dry powder, obtained after removal of the ether, was finally extracted with 16 ml of physiologic saline at pH 7.2 and centrifuged. The clear supernatant was used as antigen. Rabbit red cells were washed 3 times in saline and then subjected to the treatment described above. Rabbit plasma obtained from healthy animals was stored at −20° C and diluted in 3 parts of saline before use. *Hyperimmune serum.* Horses were immunized by subcutaneous inoculations of bone-marrow antigen as described previously(1). Two sera were available: The horse Nobert treated with a total amount of 21.3 g (dry powder) during a period of 8 months and Plym immunized with 32.75 g in the course of 4 months. *Diffusion medium.* The same medium was used as previously described (9). *Antigen analysis.* Ouchterlony's gel diffusion method(2-8) for serological analysis of precipitating antigen-antibody systems was used in combination with the inhibition method as described previously(9). This latter method was employed because of the antigenic complexity of the systems under study. By addition of one particular antigenic component to the medium the appearance of a precipitate corresponding to this antigen could be inhibited. It was thus possible, by adding extract of a certain organ to the medium, to suppress the formation of all precipitation lines belonging to this particular antigenic system. Inhibiting concentrations in the medium were established in either of two ways: a) by adding the antigen to the agar before pouring the plates or b) by diffusion from the basins. The former method was applied only when normal rabbit plasma was used as inhibiting factor. Plasma was added to a concentration of 25%, replacing equal amounts of saline. In the latter method the basins were filled on 5 consecutive days with the inhibitory preparation and the liquid allowed to diffuse completely into the gel(9).

TABLE I. Precipitation Spectrum of Antigens Tested in Diffusion Plates Inhibited with Rabbit Plasma.

Antigens	No. and character of precipitates
Bone-marrow	++++
Liver	++(+)
Spleen	+++
Lung	++(+)
Adrenal	++
Red cells	—
Plasma	—

+ = a strong line; (+) = a weak line; — = no line.

Experimental. Preliminary studies with the Nobert serum indicated that the bone-marrow of rabbit contains antigenic components, which are not demonstrable in plasma from the same species. To confirm this and to study the distribution of the non-humoral antigens in other tissues the following experiments were set up using serum from Plym. Bone-marrow, liver, spleen, lung, adrenal, red cells and plasma from rabbit were tested against anti-bone-marrow serum in diffusion plates inhibited with rabbit plasma. Efficacy of inhibition was shown by the fact that rabbit plasma gave rise to no lines of precipitate. Nor did the red cells. With the other preparations clearcut lines were obtained in varying numbers (Table I).

To check whether the extracts contained other antigens with precipitation optima outside the range of concentrations established, all extracts were tested also in dilutions of 1/5, 1/25 and 1/125. No further lines of precipitate were seen. Attempts were now made to identify the components in the different organs.

In 30 diffusion plates, inhibited as above, each extract was tested against each one of the others and the patterns of precipitation were compared. All extracts were also tested against rabbit plasma as a control. In most cases it was possible to differentiate between

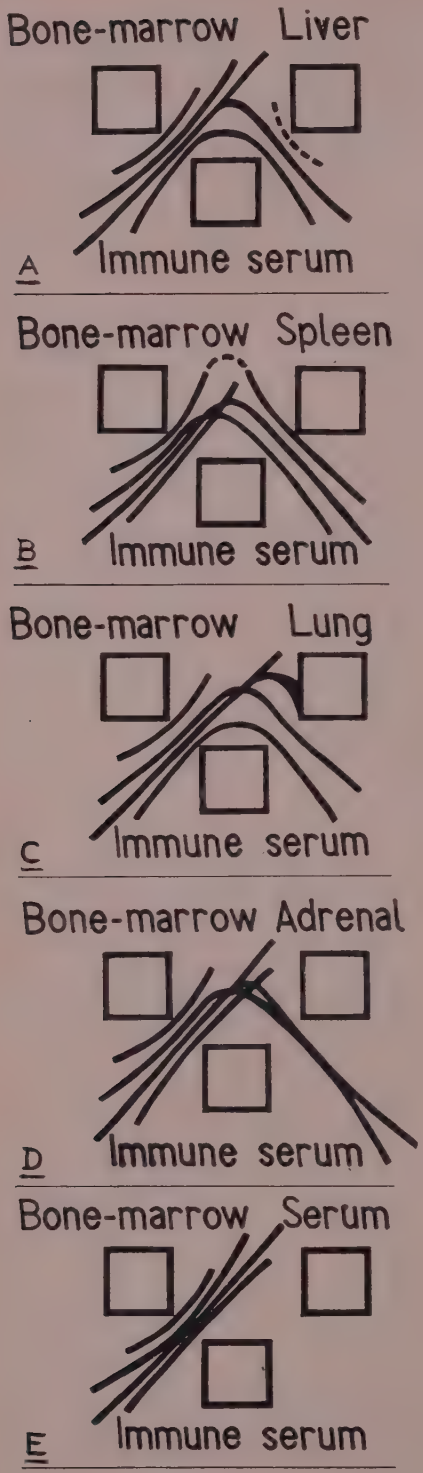

FIG. 1. A to E. Tracings of precipitation patterns in diffusion plates inhibited with normal rabbit plasma. The squares indicate the basins serving as diffusion centers of the reactants: a horse-rabbit bone-marrow hyperimmune serum and the diverse antigens as indicated in the figures.
N.B. Upper right reagent of Fig. 1E should read plasma instead of serum.

the various components and by means of the reactions of identity(5,8) to study the composition of different extracts. In some cases, however, fusion of the individual lines made such an analysis difficult or impossible. It was found that a better resolution of the precipitation patterns was obtained on dilution of the extracts. By means of this procedure clearcut results were obtainable. In all, 4 different precipitates could be distinguished, designated in the following as A, B, C and D. In Fig. 1 tracings of the different patterns are reproduced. From these it is obvious that the bone-marrow extract contained all 4 components. In the spleen A, B and C were present but not D. Liver and adrenal contained a substance designated B_1 giving a reaction of partial identity with B. Traces of B_1 were also found in the lung together with C and D. Besides B_1 the liver contained D and traces of C and in the adrenal C was found in large amounts. It was also interesting to note that the bone-marrow contained more B than the spleen but the spleen on the other hand more C than the bone-marrow.

If the statements made were correct one should expect the different extracts to inhibit precipitation to a degree corresponding to their content of antigens as shown above. For instance the bone-marrow should inhibit all lines, while spleen extract, containing only A, B and C, should leave the D-precipitate, present in bone-marrow, liver and lung, intact. The latter extracts should, therefore, give a single line in a medium inhibited with spleen extract.

Using bone-marrow, liver, spleen, lung, adrenal and rabbit plasma 6 groups of diffusion plates, totalling 162, were inhibited by the method (b). (See Methods) All plates in each group were inhibited with one of the extracts. Thus the plates of the first group were inhibited with bone-marrow, those of the second group with liver and so on. In each

TABLE II. Results of Cross-Inhibition Tests. The composition of antigens, as suggested by the analysis illustrated in Fig. 1, is indicated by the letters in the top row and the left column under the respective headings. The precipitation patterns obtained in cross-inhibition and cross-precipitation tests are indicated by the corresponding letters.

Antigens used as inhibitors	Antigens tested against anti-bone-marrow serum					
	Bone-marrow A,B,C,D	Liver B_1,(C),D	Spleen A,B,C	Lung (B_1),C,D	Adrenal B_1,C	Plasma —
Plasma —	A,B,C,D	B_1,(C),D	A,B,C	(B_1),C,D	B_1,C	—
Liver B_1,(C),D	A,B,C	—	A,B,C	C	C	(±)
Bone-marrow A,B,C,D	—	—	—	—	—	(±)
Spleen A,B,C	D	D	—	D	—	(±)
Lung (B_1),C,D	A,B	- -	A,B	—	(B_1)	(±)
Adrenal B_1,C	A,B,(D)	D	A,B	D	—	(±)

group pairs of extracts were tested in all combinations. As expected no precipitates appeared in plates inhibited with bone-marrow extract. As a whole the results confirmed entirely the previous observations (Table II.).

In a special control series normal horse serum was compared to the hyperimmune serum against all antigenic preparations. On no occasion did the normal horse serum give rise to any visible reaction.

Discussion. The appearance of multiple lines has been explained as phenomena of the type of Liesegang rings. However, the selective removal of the lines, one by one by specific inhibition, makes the assumption of the Liesegang phenomena as explanation of the multiplicity of the lines invalid. Therefore every single line has to be considered as a reaction between one particular antigen and its corresponding antibody. Thus, by means of Ouchterlony's gel diffusion method combined with a special inhibition technic it was possible to demonstrate the presence of 4 antigenic bone-marrow components and to examine their distribution in liver, spleen, lung and adrenal. None of the components could be demonstrated in normal rabbit blood indicating that they are probably cellular constituents not appearing in the circulating blood in appreciable concentrations. Different organs seem to share certain common non-humoral antigens. The actual pattern, however, differs from one organ to another.

None of these tissue antigens were bone-marrow specific in the strict sense of the word. However, the complete set of antigens was found only in the bone-marrow. The spleen containing three antigens comes next; an interesting fact with regard to the role played by this organ in hemopoiesis.

It might be assumed that there is a connection between the tissue antigens demonstrated and the serious anti-hemopoietic biological effect of anti-bone-marrow serum described in a previous paper(1). However, in attempts to explain the biological effect of anti-bone-marrow serum it must be borne in mind that there might also exist humoral antigenic substances, intrinsically connected with the hemopoiesis. Such substances, if any, could not be demonstrated by means of the method used, as the serological reactions of humoral antigens were either inhibited or so complex that a differentiation was impossible.

Summary and conclusions. 1. In a previous paper it was described how serious damage to the hemopoiesis could be produced in rabbits by injections of horse antiserum to rabbit bone-marrow. 2. As a basis for the present study it was presumed that the hemopoietic tissue contains several antigenic components, some of which may be connected with function or structure of the bloodforming system. 3. Using a special serological method, four different non-humoral antigenic components were demonstrated in the bone-marrow. None of these components was strictly specific to this organ. They were found also in liver, spleen, lung and adrenal although none of these organs contained the complete set of these antigens. Of the organs examined the spleen came closest to the bone-marrow in respect of antigenic structure. 4. It is suggested that the biological effect of the anti-bone-marrow hyperimmune serum is connected with a reaction between serum anti-

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Electron Microscopy of Euglobulin from a Case of Hyperglobulinemia.* (19366)

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The appearance of proteins, not present in normal serum in demonstrable concentrations, has been described for a number of pathologic conditions. Bence Jones protein in myeloma is a well known example. Waldenström(1,2) described a special type of hyperglobulinemia regarded by him as an independent syndrome and designated as "essential" hyperglobulinemia. Pedersen and Waldenström(3) studied the proteins in hyperglobulinemic sera and found as the most remarkable feature a fast sedimenting globulin component the molecular weight of which was estimated at about one million. Electrophoretically this macroglobulin migrated with the $\beta 2$ globulin. Serum showed a very high viscosity and in one case spontaneous congelation at temperatures below $+7°C$ was observed. Waldenström, discussing this phenomenon, tentatively assumes an elongated shape of the globulin molecules and advances also a hypothesis on the viral nature of the macroglobulin.

Since one of us (H.M.) had an opportunity to examine a similar case it seemed of interest to attempt the application of electron microscopy in the study of the pathologic protein in order to obtain some additional information about its properties.

* We wish to acknowledge our indebtedness to Dr. T. Caspersson and Dr. K. G. Thorsson of the Nobel Institute for Cell Research for the facilities made available to us and for their kind aid and interest.

Case history. A 78-year male admitted to Örebro county hospital September 1947, diagnosed anemia. He had suffered from repeated profuse bleedings from the nose. Examination disclosed a pronounced anemia and a very high E.S.R. (167 mm/1 hr). The former gel reaction was strongly positive on account of which the serum proteins were analyzed. The total protein content was 12%. By electrophoresis a considerable increase in the amount of γ globulin was found. The viscosity was very high, $\frac{\tau_i}{\tau_{i0}} = 5.7$ at 37°C, 13.3 at 13°C (J. Waldenström). On storage at $+4°C$ the serum congealed spontaneously. Euglobulin reaction strongly positive. Sedimentation analysis revealed the presence of a macroglobulin component with a sedimentation constant of $s_{20} = 19$ S (Pedersen). X-ray examination did not disclose any skeletal abnormalities. The urine was free of protein. On sternal puncture large numbers of small lymphocytes but only 0.4% plasma cells were found. Differential count of blood white cells showed 50% lymphocytes and no abnormal cells. Axillary and inguinal lymph nodes were moderately enlarged. Biopsy showed diffuse proliferation of lymphatic parenchyma with the follicular structure maintained: the sinus endothelium swollen and proliferating: catarrhal lymphadenitis without signs of a specific process (Behring). The patient showed a hemorrhagic diathesis with epistaxis and ap-

pearance of petechiae after application of a tourniquet on the arm. Thrombocyte count 70,000; bleeding time 8 minutes; coagulation time 5½ minutes; prothrombin value normal; fibrinogen 0.57%; serum vit. C normal. Anemia remained largely unchanged in spite of repeated blood transfusions and treatment with liver and folic acid. Erythrocyte sedimentation rate and hyperglobulinemia remained likewise unchanged during about 2 years. No additional symptoms indicating myeloma or leukemia were observed during this period. The patient died in his home in July 1949. Autopsy not performed.

Experimental. Serum samples were obtained on 2 occasions and stored in the refrigerator at +4°C. The euglobulin fraction was separated by dilution with 19 volumes of distilled water. A voluminous glassy, gelatinous precipitate was formed, adherent to the walls of the vessel. After centrifugation the supernate was discarded and the precipitate redissolved in the original volume of 0.9% sodium chloride solution. The procedure was repeated three times and the final precipitate redissolved in 1.25 times the original volume of saline. The solution was centrifuged for 30 minutes at 10000 r.p.m. in a Servall angle centrifuge. The inconsiderable amount of sedimentable material was discarded. The slightly opalescent solution was stored in the refrigerator until used. Immediately before use it was recentrifuged as above.

In a series of dilutions with different salt concentrations the rate of formation of a precipitate was studied. As the most suitable for the present purposes a concentration of 0.074% sodium chloride was chosen. At this level of electrolyte content a slight increase in opalescence was noted after a couple of minutes.

Three small test tubes received each 0.1 ml of a 4% osmium tetroxide solution. The macroglobulin solution was rapidly diluted with distilled water to the suitable salt concentration and 0.1 ml samples removed after 0, 2, and 15 minutes and immediately mixed with the osmic acid solutions. After fixation for 15 minutes, drops were mounted on collodion screens. Excess fluid was removed after 5 minutes and the screens were dried under an electric bulb. After drying they were washed repeatedly with distilled water and again dried, shadow-cast with palladium-platinum and examined in an RCA EMU electron microscope.

FIG. 1. Preparation of euglobulin obtained by fixation with osmium tetroxide immediately after incitation of precipitation. Shadowcast with palladium-platinum under an angle of 5°. Electron-optic magnification 6000 ×. Final magnification 17000 ×.

FIG. 2. The same preparation 2 min after incitation of precipitation.

Fig. 1-3 are representative micrographs of the specimens removed after 0, 2, and 15 minutes, respectively. It is of interest to note that the size of the aggregates is rather uniform in each individual specimen but shows a pronounced increase with the time after incitation of precipitation. This fact seems to indicate that fixation with osmic acid inter-

FIG. 3. The same preparation 15 min after incitation of precipitation.

rupts the formation of aggregates or at least slows down the process sufficiently to make a registration of the different stages possible.

The surface relief of the aggregates does not show but their contours suggest a granulated structure, although it is hardly possible to draw any conclusions regarding the size or shape of the individual molecules in the aggregates. However, a scrutiny of the micrographs reveals the presence of other structures besides the aggregates, *viz.*, small practically spherical bodies measuring ca. 120-180 Å. In the 0-minute specimen they are very numerous and not easily distinguishable from a mere coarseness of the carrier film. With the growth of the aggregates they decreased considerably in number and appear, in the 15-minute specimen, scarce and evenly distributed over the membrane. It would seem, therefore, that these structures represent particles present in the specimen and not only a rough surface of the collodion film. The obvious inverse correlation between the size of the aggregates and the number of small particles indicates that the larger structures are formed by aggregation of the latter. The size of the small particles is not incompatible with the assumption that they represent molecules of the macroglobulin.

Summary. A case of anemia combined with hyperglobulinemia is described. In the serum the presence of a macroglobulin with an estimated molecular weight of about one million was demonstrated. Electron micrographs of the partially purified macroglobulin under certain experimental conditions show the presence of spherical particles measuring approximately 120-180 Å.

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The Neuromuscular Mechanism of Alkalotic and Hypocalcemic Tetany.* (19367)

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The motor phenomena which characterize tetany consist of muscle fasciculations and tonic and clonic muscle spasms. Tetany is usually caused by a reduction in the concentration of ionized blood calcium or by an elevation in blood pH. Either of these alterations in the milieu interne apparently diminishes the stability of the nerve cell membrane, which diminution is reflected in an increased electrical excitability, altered afterpotentials and a reduced capacity of accommodation(1,2). At a critical level, iterative spontaneous discharges occur which are propagated along the nerve fiber(1,3). While

* These results were presented at meeting of American Physiological Society, Salt Lake City, Utah, Sept. 7, 1951.

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most of these observations have been made on peripheral nerves, other neural structures have been demonstrated to discharge spontaneously in hypocalcemic and alkalotic states. Bronk and associates(4) have shown this to be true for sympathetic ganglion cells. Kuffler(5) found that the peripheral nerve terminations in the motor end plate display spontaneous activity in hypocalcemic states. Very little is known of the influence of hypocalcemic and alkalotic states on neurons within the central nervous system. The question of what part or parts of the neuromuscular system are the primary site or sites of irritation in tetany is still incompletely answered. The present investigation is concerned with the analysis of this problem.

Material and methods. Eleven cats and one dog anesthetized with sodium pentobarbital (35 mg/kg) were employed in this study. Artificial respiration was provided by a Harvard type respirator pump through a tracheal cannula. Alkalosis was produced by continuous intravenous infusion of an 8% solution of sodium bicarbonate or a 5% solution of sodium carbonate. Venous blood was collected under mineral oil in a hypodermic syringe from time to time and the pH determined with the Beckman glass electrode pH meter. Hypocalcemia was produced by venoclysis of a phosphate buffer with a pH of approximately 7.4 (3 g of monobasic sodium phosphate, 30 g of dibasic sodium phosphate in 600 cc of solution). The rate of administration of these solutions was adjusted so that manifest tetany was present in from 15 minutes to 30 minutes after venoclysis was begun. The influx of solution was then slowed so as to maintain a relatively steady state of tetany until the termination of the experiment. In the analysis of the neuromuscular mechanism of tetany in these experiments it was found necessary only to section selected peripheral nerves and to employ a myoneural blocking agent, although other procedures such as spinal cord transection and dorsal root section had been contemplated. The hypoglossal nerve was dissected out in five cats at the level of the hyoid bone in the floor of the mouth and loosely ligated. The brachial plexus was exposed through a dorsal approach and this, too, was loosely ligated in 5 cats and one dog. After tetany was present, these nerves were cut and the effects on the tongue and forelimb musculature observed. In these and in other experiments the direct effect of alkalosis and hypocalcemia on muscles was observed by intravenous administration of d-tubocurarine (Squibb) in doses greater than sufficient to block transmission of nerve impulses at the myoneural junction.

Results. The objective manifestations of tetany in cats and the one dog were identical whether produced by alkalosis or by hypocalcemia. Muscle fasciculations appeared first and were most easily seen early in the tongue. These soon spread to involve other muscles. In the alkalosis series, the blood pH at the onset of fasciculations ranged from 7.65 to 7.80. Tonic muscle spasms were observed usually soon after the onset of fasciculations. The muscles of mastication and the forelimb muscles were the first to be so affected. Tonic spasm of the former muscles produced a firm elevation of the mandible. Tonic spasm of the latter muscles produced a sustained flexion at the wrist joint and in more extreme cases also an extension at the other joints, although the posture of the tonically contracted limb differed somewhat in different animals. The hindlimb and trunk musculature participated in the tonic spasms only in extreme tetany, which was induced by administering the phosphate or alkaline solution at a rapid rate after tetany was already present. This was a hazardous procedure, however, for cardiac arrest frequently supervened. At no time were clonic spasms observed.

Hypoglossal nerve section. The hypoglossal nerve was cut unilaterally proximal to its point of entry into the tongue in 5 cats after tetany was produced. Fasciculations were present in the tongue at the time of section. In three animals, there occurred a complete or partial cessation of muscle fasciculations in the ipsilateral half of the tongue. After a few seconds, fasciculations reappeared and shortly thereafter there was no perceptible difference in motor activity between the 2 halves of the tongue. In the remaining two experiments,

section of the hypoglossal nerve produced no alterations in the muscle fasciculations at any time.

Brachial plexus section. In 6 experiments the brachial plexus was cut on one side after muscle fasciculations and tonic spasms were present in the forelimbs. In 2 animals there occurred a moderate relaxation of the tonic spasm which lasted for several seconds and then was followed by a return to the previous state, the motor activity being identical in the experimental and control forelimb. In a third animal, a temporary exaggeration of the tonic spasm occurred upon cutting the brachial plexus. In another 2, section of the brachial plexus did not alter the muscle spasms of the ipsilateral forelimb. Fasciculations behaved as the tonic spasms in all these experiments.

d-Tubocurarine injection. In four experiments, d-tubocurarine was injected into the femoral vein when tetany was marked. Within a few seconds, complete cessation of all motor phenomena occurred, and persisted even after the rate of administration of phosphate or alkaline solution was increased.

Discussion. The reports in the literature dealing with this problem are frequently conflicting. Thus, some investigators(6-10) found that transection of the spinal cord at the thoracic level abolished tetany in the hindlimbs, whereas others(11,12) claimed that this procedure had no effect on tetany. Paton *et al.*(6) reported that section of the peripheral nerve abolished all manifestations of tetany in the denervated limb. West(12), however, found that this procedure, and deafferentation by dorsal nerve root section as well, blocked the tonic and clonic muscle spasms of tetany but that muscle fasciculations still occurred. The latter investigator concluded that the tonic and clonic muscle spasms in tetany depend upon the integrity of the spinal reflex arc and that the muscle fasciculations result from a direct action of the blood on the muscle fibers. The above-mentioned discrepancies in results may be attributed to two factors: the method of producing tetany, and the use of the hindlimb musculature. These investigators, with one exception(10) produced tetany by extirpation of the parathyroid glands. Parathyroidectomized animals have periods of manifest tetany alternating with latent tetany. Furthermore, they may rapidly become resistant to the hypoparathyroid state(13). This form of tetany is evidently difficult to control or regulate. The second factor, perhaps even more significant than the first, is the muscle groups singled out for the study of neuromuscular mechanism of tetany. The hindlimb musculature of mammals is relatively resistant to tetany as compared with the forelimb muscles. In spite of this, however, previous studies have employed the hindlimb muscles in analyzing the role of the nervous system. Negative results in such studies, *i.e.*, failure to observe manifestations of tetany, are not necessarily conclusive. Positive results when recorded are significant.

In this study, both of these factors were taken into consideration and the experiments designed to avoid these pitfalls. The experimental production of acute hypocalcemic and alkalotic tetany by continuous intravenous infusion of phosphate and sodium carbonate and bicarbonate solutions is a reliable method which permits minute to minute control of the severity of tetany merely by regulating the influx of solution. The muscles of the tongue and forelimbs were selected for the analysis of neural mechanism of tetany since it was these muscle groups that were found to be most responsive in tetany. The results of the present investigation show clearly that not only the fasciculations but also the tonic spasms produced in alkalotic and hypocalcemic tetany can still occur in muscles shortly after section of their nerves. West's(12) failure to observe tonic spasms in acutely denervated muscles and in muscles deafferented by dorsal nerve root section can be attributed to his employment of the hindlimb muscles of parathyroidectomized dogs. The complete and enduring abolition of all motor activity in tetany which follows the administration of d-tubocurarine, a myoneural blocking agent, eliminates the possibility of a direct activation of muscle fibers in tetany. This is consistent with a report of Hartridge and West(14) that subparalytic doses of curare abolish parathyroid tetany in dogs. In spite of this finding, however, West(12) in a subsequent re-

port inferred that muscle fasciculations result from a direct action of the blood on the muscle fibers.

Under the conditions of these experiments, it is evident that the fasciculations and tonic spasms occasioned by alkalosis and hypocalcemia can occur as the result of spontaneous iterative discharges arising in peripheral motor nerve fibers or their terminations in the motor end plates. These results cannot, of course, preclude the possibility of the participation of neurons within the central nervous system, or of sensory neurons in spinal and perhaps even supraspinal reflex arcs. In fact, there is considerable evidence that sensory fibers also are irritated and discharge repetitively in human tetany(2). However, our results strongly suggest that activation of peripheral motor nerve fibers or terminations account for the motor manifestations of tetany whether caused by an elevation in blood pH or a reduction in ionized blood calcium. This view is supported by Kugelberg's(15) finding in one human subject with inveterate hypocalcemic tetany that impulses occurring in the fourth interosseous muscle of the hand and recorded electromyographically continued unaltered after apparently effective procaine block of the ulnar nerve at the elbow.

Summary. 1. Tetany was produced acutely in eleven cats and one dog with solutions of sodium bicarbonate, sodium carbonate and a phosphate buffer administered by venoclysis. Fasciculations and tonic spasms involving especially the head and forelimb musculature characterized this form of tetany. Clonic spasms were were not seen in anesthetized animals. 2. Section of the hypoglossal nerve did not significantly influence the fasciculations of the tongue musculature. Section of the brachial plexus did not significantly influence the fasciculations or tonic spasms of the forelimb musculature. 3. Paralytic doses of d-tubocurarine blocked all motor manifestations of tetany. 4. It was concluded that all of the motor phenomena characterizing alkalotic and hypocalcemic tetany could result from the spontaneous iterative discharges occurring in peripheral motor nerve fibers or their terminations.

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Some Factors Influencing Production of Ulcers in the Shay Rat. (19368)

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In our experience, the incidence of ulcers in the Shay rat varied from 100% to 25%. There are no apparent reasons for this variation, and some of the complicating, but significant, observations are recorded in this paper. Recently, Madden *et al.*(1) in a critical study of the Shay procedure concluded that the method is valuable for the study of gastric secretion and of less value for the study of the ulceration mechanism. We have used

TABLE I. Effect of Hematuria and Pigmented Urine on Production of Ulcers.

	No. of animals	Rating					Ulcer index
		0	1	2	3	4	
Hematuria	119	69	31	14	2	3	65
Pigmented urine	55	25	19	6	4	1	85
Negative urine	864	159	140	117	86	362	241

The rating and the calculation of the Ulcer Index were as follows: 0, no visible changes; 1, incipient ulcerations, few punctate ulcers; 2, many small or one large ulcer; 3, deep ulcers with necrosis; 4, perforations of gastric wall or esophagus perforation and death before 8 hr, massive ulcerations without perforation. The Ulcer Index is the sum of each rating times 100, divided by the number of animals. Maximum index, therefore, is 400.

the method extensively and recently summarized our observations(2) on a possible role of the metabolism of certain salts on the etiology of Shay rat ulcers.

The present paper is based on 4 years experience and the use of many thousands of Shay rats. We wish to record here data concerning the incidence of ulcerations, perforations, volume of gastric contents, acidity, etc., obtained from 1038 animals. The incidence of other complications, *i.e.*, hematuria and pigmented urines, suggesting further very interesting studies is noted.

Methods. Male Wistar rats of 130-150 g weight were starved for 70 hours, with access to water, and treated using the original Shay technic(3). They were sacrificed after 8 hours, a number of them dying before that time. The system of rating and calculation of the ulcer index is given at the bottom of Table II. In most cases, analysis of the stomach juice in relation to the ulcer rating was also made.

Results and discussion. Out of 1038 Shay rats, 119 showed hematuria, 55 had pigmented urine. The effect of the development of these conditions on production of ulcers is shown in Table I.

As an indication of the frequency of esophagus ulcerations and perforations, of 200 consecutive animals which did not develop hematuria, a clear esophagus was found in 120, ulcerations in 35, and perforations in 45 animals. Data on gastric acidity and volume of stomach liquid are tabulated in Table II. With higher ulcerations the tissue debris partly buffered the acidity, but on the whole, the greater degree of ulceration was accompanied by lower pH values.

It is apparent from Table I that the occurrence of hematuria or pigmented urine greatly reduces the degree of ulceration. It appears plausible that the greater number of rats with 0 rating is due perhaps to an incipient pigment disturbance, not discerned by us. The incidence of both these disturbances varied greatly with the batch of animals received. Sometimes, almost 50% of the animals on test developed these disturbances; at other times, there were none, and the ulcer index reached the maximum of 400. Most likely the preoperative diet plays a major role, for when APF was added to the preoperative diet, the incidence of hematuria and pigmented urines were reduced with a corresponding increase in the ulcer index.

In the hematuric animals the presence of blood was plainly visible, often with congestion of the kidneys. The benzidine reaction and the presence of erythrocytes were demonstrated. The pigmented urines (we could not yet determine the nature of the pigment) con-

TABLE II.

Rating	No. of animals	Median pH	No. of animals	Median vol of gastric liquid (ml)
0	92	3	91	6
1	98	2.5	96	8
2	76	2	74	9
3	42	2.5	36	8.5
4	72	2.5	18	6.5

TABLE III. Effect of Pre-Operative Diet.

	No. of animals	% 0 rating	% hematuria
No APF added	347	34.6	18.3
APF* added	474	15.8	9.3

* Aureofac, Lederle.

tain some red cells, show a negative reaction for bile pigments, and a maximum absorption at 3700 Å, not shown by the normally colored urines. It is possible that hematuria and pigmented urine development are an expression of the same metabolic disturbance, which is responsible for the failure of these animals to develop ulcers. The role of the above pigments, or pigment derivatives is now under investigation.

Summary. The paper presents data on the degree of ulceration developed in a large number of rats treated according to the Shay technic, showing the incidence of esophageal changes, stomach acidity, and gastric liquid volume. The incidence of hematuria and pigmented urines is emphasized in their possible relationship to the etiology of Shay rat ulcers, as is the effect of preoperative diet in reducing hematuria and pigmented urines and increasing the incidence of ulceration.

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Effect of Oral Neomycin on Normal Intestinal Flora of Dogs and Man. (19369)

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Neomycin,* an antibiotic isolated from *Streptomyces fradiae* by Waksman and Lechevalier(1), has been reported to be effective *in vitro* and *in vivo* against a wide variety of gram positive and gram negative bacteria, including species resistant to other antibiotics (2-5). As the nephrotoxic and ototoxic effects of the drug(6) preclude for the present its parenteral administration, only the action after oral administration was investigated. The drug is so poorly absorbed from the gut that it is recovered nearly quantitatively and unchanged in the feces(4). This report deals with cultural data on the fecal flora after oral administration to man and to dogs.

In vitro studies. Seventy strains (*E. coli,* 10; *A. aerogenes,* 10; *Pseudomonas aeruginosa,* 10; *B. proteus vulgaris,* 10; hemolytic *Staphylococcus aureus,* 10; beta hemolytic streptococcus, 10; enterococcus, 5; and *Clostridium welchii,* 5), freshly isolated from human feces, were tested for sensitivity *in vitro* by a twofold tube serial dilution method, the concentrations of neomycin ranged from 200.0 to 0.006 μg per cc of nutrient broth. Each tube was inoculated with a bacterial suspension of 5,000-10,000 bacteria obtained by diluting an 18-24 hour broth culture of the particular strain. The lowest concentration of neomycin in μg/cc that showed no visible growth after 48 hours incubation at 37°C and did not yield living organisms in subculture was considered to be the bactericidal titer. *Comment.* In reading after 24 or 48 hours of incubation a series of tubes containing diminishing concentrations of neomycin, the last clear tube is followed by a tube just as cloudy as the control tube. In plating out this last clear tube there is no growth in the subculture. This indicates that *in vitro* neomycin has no bacteriostatic range and that the action of the drug is bactericidal—in sharp contrast to aureomycin which has a broad bacteriostatic range(7).

* The neomycin was provided by Dr. Earl L. Burbidge of Upjohn Co., Kalamazoo, Mich.

TABLE I. Bactericidal Titer of Neomycin in μg/ml in 48-Hr Nutrient Broth Cultures.

Organism	No. of strains	200	25	12.5	6.2	3.2	1.6	.8	.4	.2	.1	.05	.025
E. coli	10	—	—	1	—	—	—	1	—	—	—	1	7
A. aerogenes	10	—	—	—	—	2	1	2	—	4	—	1	—
Pseudomonas	10	—	—	—	1	1	1	2	—	3	2	—	—
B. proteus vulgaris	10	—	1	1	2	1	2	2	—	1	—	—	—
Staphylococcus aureus hemolyticus	10	—	—	—	1	—	1	2	—	1	—	2	3
β streptococcus hemolyticus	10	—	—	—	3	1	1	2	2	—	—	1	—
Enterococci	5	—	1	3	—	1	—	—	—	—	—	—	—
Clostridium welchii	5	5	—	—	—	—	—	—	—	—	—	—	—

Results. The results (Table I) confirm the reported data(2-4) that strains of *E. coli, A. aerogenes, Pseudomonas aeruginosa,* and hemolytic *Staphylococcus aureus* are very sensitive to neomycin, strains of *B. proteus* are moderately sensitive, enterococci are moderately resistant, and *Clostridium welchii* completely resistant to the drug. The strains of hemolytic streptococci herein reported were moderately sensitive to neomycin. This is in accord with some published data(4), while other reports indicate this species to be moderately resistant to the drug(5).

Effect of oral neomycin on the normal fecal flora of dogs. Method. Each of 37 healthy mongrel dogs received 0.4 g/kg of neomycin orally each day in 4 divided doses. In the first group of 17, the drug was given for 2 days; in the second group of 10 for 4 days; and in the third group of 10 for 10 days. The dogs were allowed to eat and drink as usual during the entire period of observation. No toxic reactions were observed. The fecal flora of all dogs was examined before the drug was started, daily during administration of the drug, and for a few days thereafter. The coliform bacilli were counted by the method of Spaulding *et al.*(8).

Results. 1. *Coliform bacilli.* In the 17 dogs treated for 2 days coliform bacilli disappeared from stool cultures in 1-3 days and then reappeared in steadily increasing numbers. In 8 of 10 dogs treated for 4 days the disappearance rate and reappearance following cessation of therapy were the same. In the remaining 2 there was only a marked reduction in the coliform count during and for 1-2 days after treatment. In 9 of 10 dogs which received the drug for 10 days, coliform bacilli disappeared within 1-4 days and were markedly reduced in the 10th. However, in 4 of these dogs *E. coli* reappeared on the 5th-6th day and rapidly reached pretreatment levels. These strains were tested *in vitro* and were found to be resistant to the drug (bactericidal titer 25 to 50 μg/cc). 2. *Pseudomonas* was present initially in the stool cultures of 18 of the 37 dogs disappearing in 9 of these after 2-6 days of neomycin. There was no appreciable change in the remaining 9 dogs. 3. *Proteus vulgaris.* Strains of this species, present only in 6 of the 37 dogs prior to neomycin, disappeared in 4 after 2-3 days of treatment. 4. *Enterococci* were found initially in the stools of only 7 dogs. They disappeared in all 7 after 2-4 days and remained absent during treatment and for 1-2 days thereafter. 5. *Yeasts* were found in many stools shortly after treatment was started. When all gram negative bacilli had been eliminated the aerobic plates frequently showed pure cultures of yeasts. However, they were never found in large numbers and there was no apparent increase during treatment. 6. *Clostridia* were present initially in the feces of 30 of 37 dogs. The number of colonies of these organisms on anaerobic blood plates from 24-hour thioglycollate subcultures was small to moderate, relative to the number of other fecal flora. The numbers of colonies was not appreciably reduced during treatment. Indeed, in several dogs all other bacteria were cleared from the feces and pure cultures of *Clostridia* remained. Hence *in vivo* resistance parallels the *in vitro* resistance of this species to neomycin.

Ten dogs were given a single dose of 6 g neomycin by gavage just prior to inducing hemorrhagic shock. Two died after 12 hours, 2 after 48 hours. The rest were sacrificed after 2 days. Cultures of small intestine and colon taken immediately after death showed the following: In the 2 dogs dead of shock after 12 hours, cultures from the jejunum yielded few to moderate number of colonies of *E. coli, Clostridia,* and enterococci. Cultures from the colon showed a large number of colonies of *E. coli,* a moderate number of colonies of *Clostridia* and a few colonies of enterococci. In all dogs dead or sacrificed after 48 hours cultures yielded no coliform bacilli in the jejunum or colon, but there were present moderate numbers of colonies of *Clostridia,* yeasts, and occasionally a few of *Pseudomonas.*

Comment. A single large dose of neomycin will eliminate coliform bacilli from the cultures of intestinal contents of the dog within 48 hours or less. This cannot be achieved so rapidly after oral administration of streptomycin, aureomycin, or sulfonamides(9).

Effect of oral neomycin on normal fecal flora of man. Three groups of patients without disease of the gastrointestinal tract received neomycin. In the first 8 patients, a single 6 g dose was given; in the second 8 patients a 6 g dose was given in 4 divided doses of 1.5 g every 6 hours for 24 hours; and in the third group of 9 patients, a 6 g dose was given daily for 3 days, 1.5 g every 6 hours. Two patients of the latter group had nausea or vomiting, and one had diarrhea. The pattern of the intestinal flora was determined in each case from stool cultures before treatment was started, and daily thereafter until the pretreatment status was restored. Quantitative changes in the coliform count were determined by the method of Spaulding(8).

Results. a) *Single 6 g dose—8 patients.* In 5 of the 8 patients the coliform bacilli disappeared from the cultures and remained absent for 48 hours in 4 of them. In the remaining 3 there was a marked reduction in the coliform count within 24 hours. In all 8 patients the count returned to pretreatment levels between the 4th and 6 day. Pseudomonas disappeared from the cultures in 2 patients and remained absent for 2 days thereafter. In 4 others there was no change, and in the remaining 2 none were cultured either before or after therapy. There was no appreciable change in the count of Clostridia or enterococci. b) *One and a half g every 6 hours for 1 day—8 patients.* Coliform bacilli disappeared from cultures in 24-48 hours after onset of therapy in 6 of the 8 patients, and remained absent for 24-72 hours thereafter. In the 7th patient there was only slight reduction and in the 8th no reduction in the coliform count. Clostridia, enterococci, and Pseudomonas were not significantly suppressed. c) *One and one-half g every 6 hours for 3 days—9 patients.* In all cases coliform bacilli disappeared from cultures within 24-48 hours. They remained absent for 2 days in 4 patients, for 3 days in 3, for 4 days in 1, and for 5 days in 1. Subsequently there was gradual return to pretreatment levels. Pseudomonas was suppressed after 2 days of neomycin therapy in 4 patients and remained absent from cultures for 1-2 days thereafter. The remaining 5 showed no change. Enterococci disappeared from the cultures in 3 after 2 days of neomycin administration. There was no change in 3 others. In the remaining 3 enterococci were absent before treatment was instituted. Clostridial growth was not altered in any instance.

Summary. 1. Ten random strains of each of the following species were sensitive *in vitro* (3 exceptions in 40 cultures) to 6.2 μg/cc or less of neomycin in a 48-hour nutrient broth culture: *E. coli, A. aerogenes, Pseudomonas aeruginosa, B. proteus vulgaris, Hemolytic staphylococcus aureus* and *Hemolytic streptococcus.* In 5 strains of enterococci the range of sensitivity was 25-3.2 μg neomycin/cc, while Clostridia were not sensitive at a concentration of 200 μg/cc or higher. When neomycin is effective, its antibacterial action is bactericidal and not bacteriostatic under the conditions of the test(8). 2. Oral administration of neomycin usually eliminates coliform bacilli from stool cultures in dogs and in man within 48 hours and frequently within 24 hours. Continuation of the drug for 5-6 days resulted in the appearance of resistant strains of *E. coli* in dogs. Such resistance was not

observed in 25 humans. 3. The numbers of *Enterococci, Ps. aeruginosa,* and *B. proteus vulgaris* in the stool are reduced in most cases by oral neomycin therapy. 4. Clostridia are not noticeably affected by neomycin.

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